



**Liliana Sofia Machado de Carvalho**

1º Ciclo de Estudos em Ciências Biomédicas

**Expression of Cyclooxygenase Enzymes and  
Prostaglandin E<sub>2</sub> Receptors in Inflammatory Airway  
Diseases**

Dissertação para obtenção do Grau de Mestre  
em Genética Molecular e Biomedicina

Orientador: Cesar Picado Vallès, Professor Doutor, FM/UB

Co-orientador: Jordi Roca-Ferrer, Doutor, FM/UB

Júri:

Presidente: Prof. Doutor José Paulo Nunes de Sousa Sampaio

Arguente: Prof. Doutora Susana Isabel Rodrigues dos Santos

Vogal: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves



FACULDADE DE  
CIÊNCIAS E TECNOLOGIA  
UNIVERSIDADE NOVA DE LISBOA

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# Expression of Cyclooxygenase Enzymes and Prostaglandin E<sub>2</sub> Receptors in Inflammatory Airway Diseases

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## Abstract

Aspirin-intolerant asthma (AIA) is a syndrome where the interplay between cyclooxygenase (COX) and lipoxygenase (LOX) pathways is evident and is characterized by several abnormalities in the regulation and biosynthesis of eicosanoid mediators and eicosanoid receptors. Several observations indicate the presence of a complex change in the arachidonic acid (AA) metabolism in patients who suffer from AIA. However, previous studies showed some discrepant results when upper and lower airways were analyzed, and there are no clear explanations for this. The inflammatory responses in upper airways are often associated with the presence of nasal polyps, structures never seen in the lower airways. In this study, upper and lower airways were compared, in order to verify if the multiple factors of the COX pathway are differentially regulated considering both respiratory tracts. To perform the experiments, fibroblasts from nasal mucosa (NM) and bronchial mucosa (BM) of non-asthmatic subjects undergoing corrective surgery and fibrobronchoscopy, respectively, (control group) were compared with NM, nasal polyp (NP), and BM fibroblasts isolated from non-asthmatic and AIA patients suffering from chronic rhinosinusitis (CRS) with NP. The data presented in this investigation suggest that in lower airways, the presence of aspirin intolerance does not seem to alter the expression of COX enzymes or the production of prostaglandin (PG) E<sub>2</sub>. Considering the expression of the EP receptors, the data suggest significant differences through fibroblasts from upper airways tissues. The differences observed between upper and lower airways combined with others verified in previous studies might contribute to the pathogenesis of nasal polyposis.

**Keywords:** Aspirin intolerant-asthma, cyclooxygenase, EP receptors, fibroblast, nasal polyp, prostaglandin E<sub>2</sub>, upper and lower airways.





## Resumo

A asma com intolerância à aspirina (AIA) é uma síndrome na qual existe uma estreita relação entre as vias da cicloxigenase (COX) e lipoxigenase (LOX) e é caracterizada por várias anomalias na regulação e biossíntese dos mediadores de eicosanóides e receptores de eicosanóides. Diversas observações indicam a presença de uma complexa alteração no metabolismo do ácido araquidónico (AA) em pacientes com AIA. Contudo, estudos anteriores reportaram alguns resultados discrepantes no que diz respeito às vias aéreas inferiores e superiores e não existe uma explicação clara para tal facto. A resposta inflamatória que ocorre nas vias aéreas superiores está frequentemente associada à presença de pólipos nasais, estruturas nunca observadas nas vias aéreas inferiores. Neste estudo, foram comparadas as vias aéreas superiores e inferiores, de modo a verificar se os múltiplos factores envolvidos na via da COX apresentam uma regulação diferencial em ambos os tratos respiratórios. Para a realização deste estudo foram isolados fibroblastos da mucosa nasal (NM) e mucosa brônquica (BM) de indivíduos não asmáticos submetidos a cirurgia correctiva do nariz e fibrobroncoscopia, respectivamente, (grupo controle), sendo posteriormente comparados com fibroblastos de NM, pólio nasal (NP) e BM isolados de indivíduos não asmáticos e doentes com rinosinusite crónica (CRS) e NP. Os dados apresentados sugerem que, nas vias aéreas inferiores, a intolerância à aspirina não parece alterar a expressão das enzimas COX ou a produção de prostaglandina (PG) E<sub>2</sub>. Considerando a expressão dos receptores de prostaglandina E<sub>2</sub> (EP), os resultados mostram diferenças significativas considerando fibroblastos isolados de tecidos provenientes das vias aéreas superiores. As diferenças observadas entre via superior e inferior, e tendo em conta outras diferenças verificadas em estudos anteriores, podem estar envolvidas no processo de formação de pólipos.

**Termos-chave:** Asma com intolerância à aspirina, cicloxigenase, fibroblastos, pólio nasal, prostaglandina E<sub>2</sub>, receptores EP, vias aéreas superiores e inferiores.



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## List of Abbreviations

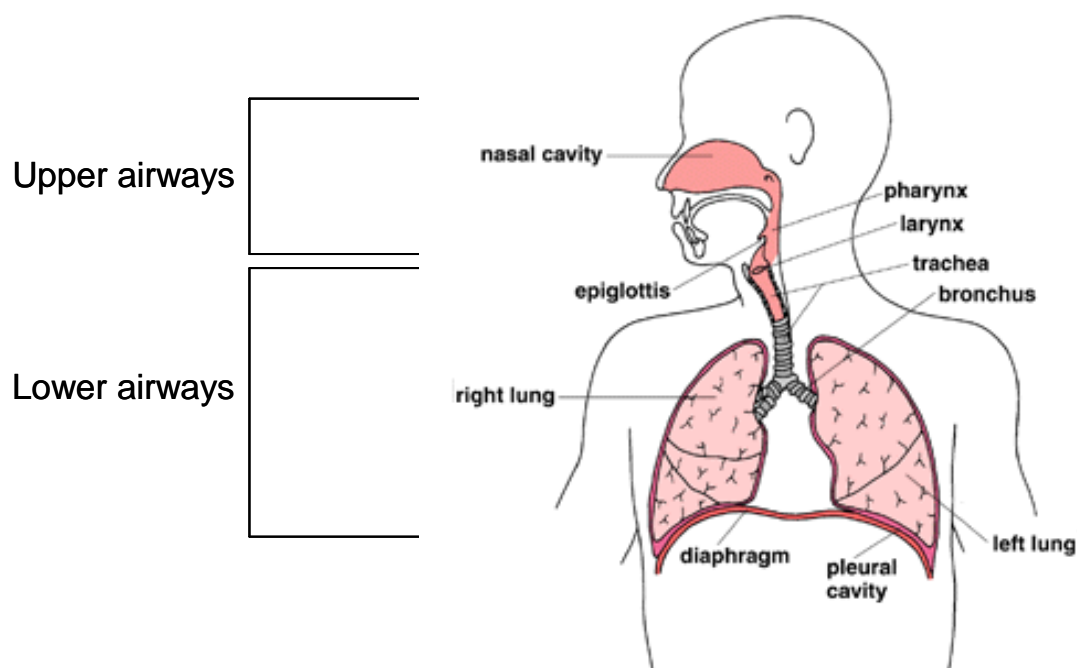
|                         |                                       |
|-------------------------|---------------------------------------|
| <b>AA</b>               | Arachidonic acid                      |
| <b>AIA</b>              | Aspirin-intolerant asthma             |
| <b>AT</b>               | Aspirin tolerant                      |
| <b>BM</b>               | Bronchial mucosa                      |
| <b>bp</b>               | Base pairs                            |
| <b>BSA</b>              | Bovine serum albumin                  |
| <b>cAMP</b>             | Cyclic adenosine 3', 5'-monophosphate |
| <b>CCD</b>              | Charge-coupled device                 |
| <b>COX</b>              | Cyclooxygenase                        |
| <b>CRS</b>              | Chronic rhinosinusitis                |
| <b>Cys</b>              | Cysteinil                             |
| <b>DAPI</b>             | 4',6-diamidino-2-phenylindole         |
| <b>ddH<sub>2</sub>O</b> | Double-distilled water                |
| <b>DMEM</b>             | Dulbecco's modified Eagle's media     |
| <b>DMSO</b>             | Dimethyl sulfoxide                    |
| <b>DNA</b>              | Deoxyribonucleic acid                 |
| <b>ECM</b>              | Extracellular matrix                  |
| <b>ELISA</b>            | Enzyme-linked immunosorbent assay     |
| <b>EDTA</b>             | Ethylenediaminetetraacetic acid       |
| <b>EMEM</b>             | Eagle's minimum essential media       |
| <b>EP</b>               | Prostaglandin E <sub>2</sub> receptor |
| <b>FBS</b>              | Fetal bovine serum                    |
| <b>Ig</b>               | Immunoglobulin                        |
| <b>IL</b>               | Interleukin                           |
| <b>kDa</b>              | Kilodalton                            |
| <b>LDS</b>              | Lithium dodecyl sulfate               |
| <b>LOX</b>              | Lipoxygenase                          |
| <b>LT</b>               | Leukotriene                           |
| <b>NM</b>               | Nasal mucosa                          |
| <b>nm</b>               | Nanometres                            |
| <b>NP</b>               | Nasal polyp                           |
| <b>NSAID</b>            | Nonsteroidal anti-inflammatory drug   |
| <b>PBS</b>              | Phosphate buffered saline             |
| <b>PCR</b>              | Polymerase chain reaction             |
| <b>PG</b>               | Prostaglandin                         |
| <b>RNA</b>              | Ribonucleic acid                      |
| <b>SDS</b>              | Sodium dodecyl sulfate                |

|             |                                 |
|-------------|---------------------------------|
| <b>SFM</b>  | Serum-free media                |
| <b>sma</b>  | Smooth muscle actin             |
| <b>TBE</b>  | TRIS/Borate/EDTA                |
| <b>TRIS</b> | tris(hydroxymethyl)aminomethane |

# 1. Introduction

## 1.1. The Airways

The main purpose of the respiratory system is to supply the body with oxygen and remove carbon dioxide, i.e., to promote gas exchange between our body and the external environment. The airway is a continuous and large structure that extends from the nasal vestibule to the alveoli. There is an imaginary line that divides the airways into two subdivisions: upper and lower airways (Figure 1.1) (Scadding and Kariyawasam, 2009). The upper airways, also known as the upper respiratory system, include the nose, the nasal cavity, the paranasal sinuses, the nasopharynx and the oropharynx. On the other hand, the lower respiratory system (lower airways) consists of the larynx, trachea, bronchi, and lungs (Jablonka and Rosenblatt, 2009).



**Figure 1.1.** Schematic representation of the respiratory system. The figure represents the two sub-divisions of the airways (adapted from <http://drraghu74.blogspot.com/2009/09/respiratory-system.html>).

The nose is an organ included in the upper airways that participates in several functions related to respiration. The major functions of the nose are to prepare the inhaled air, by filtering, warming, and moistening it before reaching the lungs. Since the nose acts as a filter and air-conditioner, it protects the lower airways. Effectively, the nose and their associated structures are the first site of allergen, microbial, and particle deposition (Scadding and Kariyawasam, 2009). Considering that, pathological

conditions of the nasal mucosa (NM) interfere with the nose functions, which can lead to increased exposure of the lower airways to allergens and subsequent airway inflammation (Braunstahl, 2011).

## **1.2. Inflammation and Airway Inflammation**

Inflammation is a protective response initiated after injury through physical damage or infection by microorganisms. This essential biological process consists in eliminating the aberrant factors, promote tissue repair/wound healing, and establish memory. Inflammation is composed by an acute phase, which involves a large number of molecular, cellular and physiological changes. The acute phase is characterized by the rapid influx of blood granulocytes (neutrophils, monocytes, and macrophages). This mechanism is responsible to the cardinal signs of acute inflammation, as redness, heat, swelling, and pain. Concluded the set of responses, inflammation is resolved and the restoration of the inflamed tissue, as well as homeostasis is reestablished. However, if defects in the process occur, inflammation will persist and become chronic, lasting for longer periods, leading to excess tissue damage (Ricciotti and FitzGerald, 2011).

Effectively, pro-inflammatory mediators generated in inflamed tissue drive to acute inflammation. However there is a systemic and local production of endogenous mediators that counter-balance the pro-inflammatory events occurred in these locals. Endogenous mediators are produced to avoid the development of chronic pathologies. For instance, lipid mediators derived from polyunsaturated fatty acids, such arachidonic acid (AA), are synthesized during the normal cell function, or after cell activation in stress conditions, in order to perform anti-inflammatory actions (Stables and Gilroy, 2011).

Diseases characterized by airway inflammation, excessive airway secretion, and airway obstruction affect a great proportion of the worldwide population. The excessive airway production of chemokines, cytokines, and growth factors in response to irritants, infectious agents, and inflammatory mediators may play an important role in the modulation of acute and chronic airway inflammation. Lipid mediators are chemical messengers that are released in response to tissue injury. These mediators may be produced by resident airway cells and by inflammatory cells or can be also altered by inflammatory cytokines (Levine, 1995).

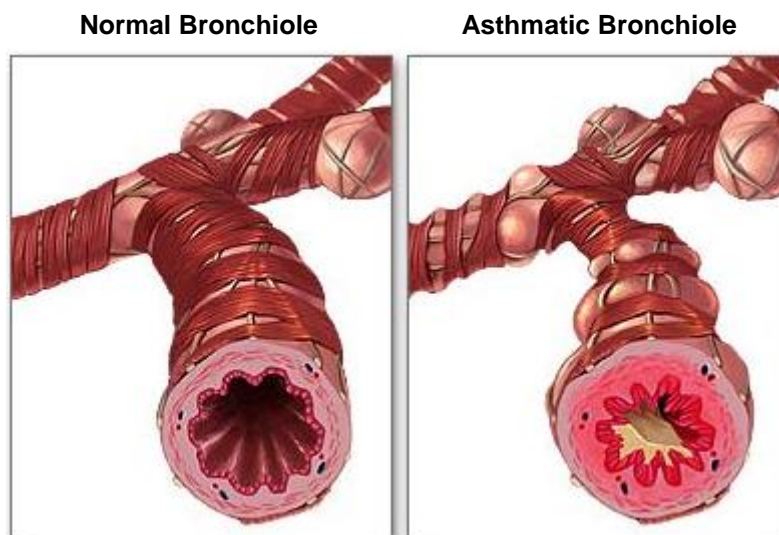
The main four signs of airway inflammation are edema (swelling), vasodilatation (redness), cellular infiltration, and pain (increased airway responsiveness). Since the airways have no pain fibers, pain is defined, in this case, as an increase in airway responsiveness (Levine, 1995).

### 1.2.1. Airway Inflammatory Diseases

#### 1.2.1.1. Asthma

Asthma is defined by the Global Initiative for Asthma as: “a *chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment*” (Muñoz-López, 2010).

Asthma is a complex multifactorial disease of the airways and is currently a worldwide problem, with an estimation of 300 million of affected (Gohil *et al.*, 2010). The disease may develop early in childhood or later on in life, and its pathogenesis seems to be associated with the interaction of several single-nucleotide polymorphism loci. The main features of asthma are airflow obstruction, chest tightness, wheezing and cough. The airflow obstruction is due mostly to smooth muscle contraction, bronchial wall edema and mucus plugging that leads to a partial or complete closure of the airways (Figure 1.2). There are many factors that can precipitate asthma attacks. These factors include allergy, viral infections, exercise, and airborne irritants, such as smoke cigarette or dusts. Asthma can be intermittent, or persistently mild, moderate or severe. The severity of the pathology varies among subjects and can change in one individual over time (O'Byrne, 2008).



**Figure 1.2.** Normal versus asthmatic bronchiole (adapted from <http://health.allrefer.com/health/asthma-normal-versus-asthmatic-bronchiole.html>).

Pathophysiologically, asthma is characterized by epithelial disruption, airway smooth muscle hypertrophy and hyperplasia, increased mucus secretion, basement membrane thickening, increased cytokine production and chronic infiltration of inflammatory cells. These changes that occur to a

structural level are often referred as remodeling, that define complex morphological changes involving all structures within the bronchial wall (Hamid and Tulic, 2007; Blake, 2006; Fireman, 2003).

Respiratory inflammation commonly affects both upper and lower respiratory tracts concurrently. Attention to the presence of asthma associated with other airway diseases, as rhinosinusitis is of importance while this condition often affects the quality of life more negatively as asthma alone (Marseglia *et al.*, 2010).

### **1.2.1.2. Chronic Rhinosinusitis and Nasal Polyposis**

Sinusitis involves inflammation of the sinus linings and rarely occurs without simultaneous rhinitis. Therefore, the usually term is a combination of both – rhinosinusitis. Rhinosinusitis is defined as an inflammatory process involving the mucosa and one or more sinuses (Scadding *et al.*, 2008). The International Classification of Diseases divides rhinosinusitis into two forms: acute and chronic, according to the duration of symptoms. According with this classification, the acute form lasts up to 12 weeks with complete resolution of symptoms. On the other hand, the chronic form that persists beyond 12 weeks is associated with significant morbidity and a lower quality of life (Fokkens *et al.*, 2005; Meltzer *et al.*, 2004). Chronic rhinosinusitis (CRS) constitutes one of the most common health care problems and had direct medical costs as well as severe impact on lower airway diseases and general health outcomes (Fokkens *et al.*, 2005).

CRS is, as asthma, a multifactorial disease and is subdivided into two classes: CRS without nasal polyposis and CRS with nasal polyposis (Ferguson, 2004). Histologically, sinusal polyps are characterized by proliferation and thickening of mucosal epithelium with focal squamous metaplasia, glandular hyperplasia, subepithelial fibrosis, and stromal edema with numerous blood vessels. The most common inflammatory cells that appear in nasal polyps (NPs) are the eosinophils, but fibroblasts, mast cells and goblet cells are also present (Fokkens *et al.*, 2005). NP and CRS are considered many times as only one disease, because the differentiation between them is very complicate and nearly impossible. CRS associated with nasal polyposis is very difficult to treat, while its etiology and pathophysiology are still unclear, and even after surgery and medical treatments, recurrences are frequent. Patients with NPs often present other related diseases, such as asthma, aspirin intolerance, sinobronchial syndrome, or cystic fibrosis (Pawankar and Nonaka, 2007).

Clinically, the diagnostic of CRS requires two or more of the following symptoms: nasal blockage, anterior or postnasal drip, facial pain or pressure, and reduced or absent sense of smell. Besides, there is an endoscopic intervention that allows document the presence or absence of inflammation (Pawankar and Nonaka, 2007).



### 1.2.1.3. Aspirin-Intolerant Asthma

Aspirin-intolerant asthma (AIA) is a distinct clinical syndrome characterized by the association of asthma, CRS, bilateral nasal polyposis, and episodes of bronchospasm precipitated by non-steroidal anti-inflammatory drugs (NSAIDs) (Stevenson and Szczeklik, 2006). The first case of AIA was reported in 1922 by Widal and collaborators, when an episode of bronchospasm was observed following the ingestion of acetyl salicylic acid (aspirin) in a subject who suffer from asthma (Varghese and Lockey, 2008). In the late 1960's, Samter and Beers described this peculiar syndrome with its clinical *triad* of asthma, nasal polyposis, and aspirin intolerance, in greater detail (Samter and Beers, 1968).

Actually, aspirin and NSAIDs are among the most widely used medication in the world. Aspirin and other NSAIDs are prescribed to treat and prevent heart diseases, as well as used to antipyretic, anti-inflammatory, and analgesic therapy. Normally, for most of people, these drugs are well tolerated, but asthmatic patients are an exception. In several patients with bronchial asthma, aspirin and other NSAIDs are capable to precipitate attacks of asthma. The severity of the attacks is variable, depending on the patient (Szczeklik and Sanak, 2006). Considering that, AIA is actually recognized to be an aggressive phenotype of airway disease that often runs a protracted course. When compared with aspirin-tolerant (AT) subjects, patients with AIA are more susceptible to experience irreversible airway flow obstruction, to suffer frequent exacerbations, as well as to be diagnosed with severe asthma (Koga *et al.*, 2006; Mascia *et al.*, 2005).

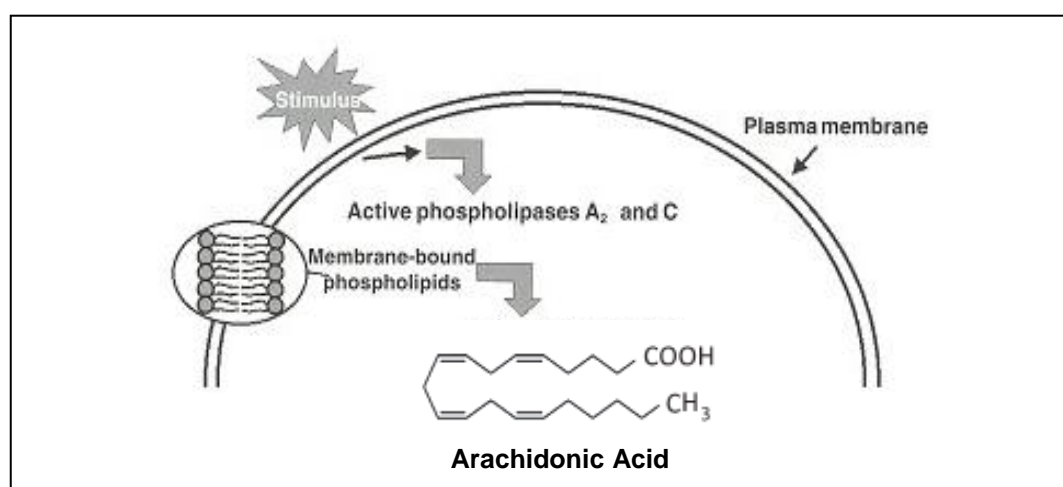
The exposure to aspirin or other NSAIDs does not initiate or perpetuate the underlying respiratory inflammatory disease. However, since the disease is ongoing, these drugs induce release or synthesis of critical mediators (Gohil *et al.*, 2010). After ingestion of aspirin or NSAIDs, patients with adverse reactions develop symptoms within 2 – 3 hours. Symptoms generally consist of bronchospasm, profuse rhinorrhea, conjunctival injection, periorbital edema, and generalized flushing (Morwood *et al.*, 2005; Szczeklik and Stevenson, 2003).

This peculiar syndrome is presents in approximately 3 to 20% of the adult asthmatic population, and is more frequent in women than in men (Gohil *et al.*, 2010). Additionally, in women the symptoms emerge normally earlier, and the disease seems to be more aggressive (Szczeklik *et al.*, 2000). Aspirin sensitivity has been reported in 35 – 52% of patients with NPs and in as many as 65% of patients who suffer from both bronchial asthma and nasal polyposis (Schiavino *et al.*, 2000). Patients with aspirin/NSAID-induced respiratory reactions often have an underlying history of asthma, nasal polyposis, and/or rhinosinusitis (Gollapudi *et al.*, 2004), although some patients may not have any predisposing diseases. Subjects who develop the aspirin *triad* typically develop persistent rhinitis in their third or fourth decade of life and, approximately 2– 5 years later, there is a progression to asthma, aspirin sensitivity, and nasal polyposis (Hamad *et al.*, 2004).

Aspirin sensitivity is not considered an immunological reaction, because the pathology did not involve an immunoglobulin (Ig) E-mediated reaction (Pfaar and Klimek, 2006). The pathological mechanism responsible for the development of AIA has not been completely elucidated. However, several observations suggest that abnormalities in the regulation of AA may be involved in the adverse response to NSAIDs in patients with this pathology (Yoshimura *et al.*, 2008; Stevenson and Szczeklik, 2006).

### 1.3. AA Metabolic Pathways

AA, a 20-carbon polyunsaturated fatty acid (20:4  $\omega$ -6), is the main eicosanoid precursor. Under normal conditions the concentration of free AA within the cells is low. Most of it is stored as part of phospholipids in the membranes of the cells (Brash, 2001). The availability of free AA is essential for the biosynthesis of eicosanoids. Therefore, this mediator is released from the phospholipid membranes by the action of various phospholipase enzymes, which are activated in response to different stimulus (physical, chemical, hormonal, cytokines, etc) (Figure 1.3). The mainly enzyme implicated in the release of AA seems to be the cytosolic phospholipase A<sub>2</sub> (Picado, 2006). When AA is released from the membrane, it is rapidly metabolized in several enzymatic and non-enzymatic pathways to yield an important family of oxygenated products, collectively termed eicosanoids, released from the source cell and act in an autocrine/paracrine manner on target cells. The three main enzymatic pathways responsible to the metabolism of AA include (1) the lipoxygenase (LOX) pathway, responsible to the formation of leukotrienes (LTs) and lipoxins, (2) the cyclooxygenase (COX) pathway, involved in the formation of prostaglandins (PGs), thromboxanes (Tx<sub>s</sub>), and prostacyclin, and (3) the cytochrome P450, responsible for the formation of epoxyeicosatrienoic and hydroxyeicosatetraenoic acids (Stables and Gilroy, 2011; Simmons *et al.*, 2004).



**Figure 1.3.** Schematic representation of AA release process. Membrane-bound phospholipids are converted to AA by the action of phospholipase enzymes, which are activated in response to external stimuli (adapted from Stratton and Alberts, 2002).

### 1.3.1. COX Pathway

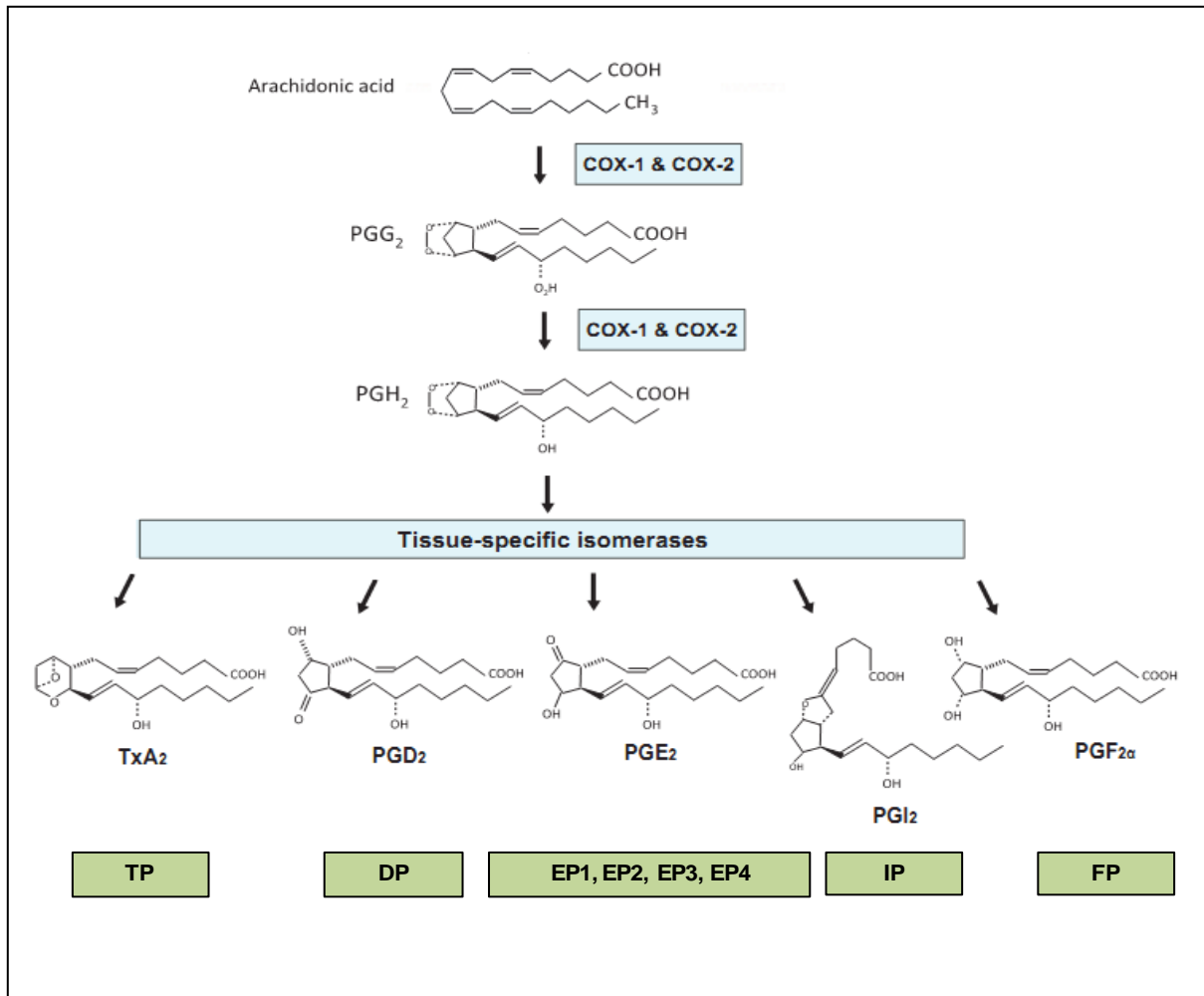
COX is a bi-functional enzyme involved in the COX pathway. COX-1 and COX-2 isoenzymes catalyze the same reactions, show approximately 60 – 65% identity in their amino acid sequence within a given species, but are encoded by two different and specific genes, located in distinct chromosomes. These enzymes are located in the luminal portion of the endoplasmic reticulum membrane and the nuclear envelope (Chandrasekharan and Simmons, 2004). COX-1 serves a number of physiologic “housekeeping” functions, such as modulation of platelet aggregation and cytoprotection in the gastrointestinal mucosa. In addition, the expression of COX-1 is developmentally regulated in many different tissues, and small changes in expression can occur after stimulation with hormones or growth factors (Rocca and FitzGerald, 2002). On the other hand, COX-2 is highly induced in macrophages, fibroblasts, vascular endothelial cells, and smooth muscle cells by various cytokines, endotoxins, growth factors, or tumor promoters. COX-2 is the more important source of prostanoid formation in inflammatory processes (Baigent and Patrono, 2003). A third form (COX-3) has also been described, although recent studies indicate that this form represents a splice variant of COX-1 that encodes a truncated protein lacking enzymatic activity (Snipes *et al.*, 2005).

AA can be metabolized by COX enzymes (Figure 1.4). Once AA is released, COX isoenzymes catalyze the cyclization of AA to form  $\text{PGH}_2$  through a two-step redox reaction: (1) AA is oxidized by the COX enzymes into the unstable intermediate  $\text{PGG}_2$  in the active site of the enzyme and then (2)  $\text{PGG}_2$  is reduced by the peroxidase activity of COX to form  $\text{PGH}_2$ .  $\text{PGH}_2$  is the precursor of several bioactive prostanoids, which are formed by the action of specialized tissue isomerases. The five prostanoids synthesized by this pathway include  $\text{PGE}_2$ , as well as  $\text{PGD}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGI}_2$ , and  $\text{TxA}_2$  (Rocca, 2006). After the synthesis, the prostanoids exit the cells in order to activate G-protein-coupled receptors or nuclear receptors in target cells. The prostanoid receptor subfamily is comprised of eight members (DP, EP1-4, FP, IP, and TP), which are classified according to the prostanoid ligand that each binds with greatest affinity (Hata and Breyer, 2004).

$\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGI}_2$ , and  $\text{TxA}_2$  are metabolites that exert their biological effects in the proximity of the sites of their synthesis, in autocrine or paracrine manner. These mediators play an important role in the inflammatory process. In inflamed tissues, their biosynthesis is significantly increased, and they contribute to the development of the main signs of acute inflammation. Moreover, during an inflammatory response, the level and profile of PG production change significantly (Ricciotti and FitzGerald, 2011).

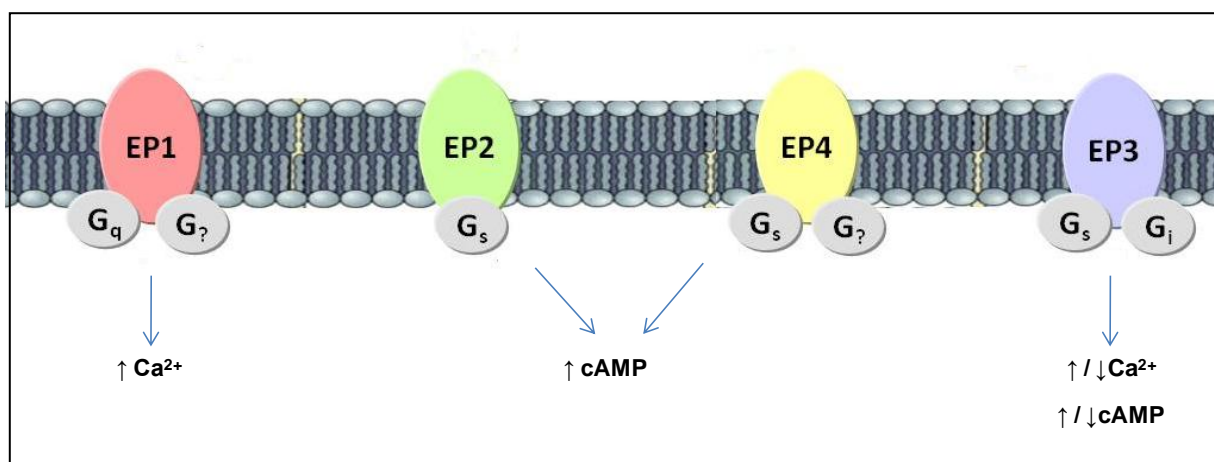
$\text{PGE}_2$  is one of the most abundant prostanoid produced in the body and exhibits versatile biological activities. Additionally, it seems to have an important role in inflammatory processes (Ricciotti and FitzGerald, 2011). As previously described, the activity of  $\text{PGE}_2$  is mediated by four receptors, termed prostaglandin  $\text{E}_2$  receptors (EP) (EP1 – EP4), which are encoded by distinct genes and have divergent amino acid sequences, but all bind  $\text{PGE}_2$  with high affinity. Thus, through the multiple receptor subtypes,  $\text{PGE}_2$  can trigger several intracellular signal transduction pathways and has diverse final

effects, which sometimes seem to be functionally opposing within the same cell or organ (Rocca, 2006). The complexity of PGE<sub>2</sub> responses is further complicated by evidence that multiple EP receptors are often co-expressed or induced in the same cell or organ. The regulation of this co-expression is still unknown, but it indicates that the response to PGE<sub>2</sub> is modulated, based on the activation of different pathways by different EP receptor subtypes (Rocca, 2006).



**Figure 1.4.** Representation of the COX pathway, showing the several prostanoids and the receptors through them act. AA is converted to PGH<sub>2</sub> through a two-step process that involves COX activity to convert AA to PGG<sub>2</sub> followed by a peroxidase reaction, mediated also by COX enzymes to produce PGH<sub>2</sub>. The formation of the several PGs is carried out by tissue-specific isomerases (adapted from Ricciotti and FitzGerald, 2011).

EP1 activates phosphatidylinositol metabolism leading to the formation of inositol triphosphate with mobilization of intracellular free calcium. EP2 and EP4 stimulate adenylate cyclase, leading to the production of cyclic adenosine 3', 5'-monophosphate (cAMP), which activates the cAMP-dependent protein kinase A. Stimulation of EP4 also activates phosphoinositide-3'-kinase. EP3 is the only receptor that possesses multiple splice variants and the different isoforms couple to multiple G proteins producing either inhibition of adenylate cyclase or stimulation of adenylate cyclase activity (Figure 1.5) (Vancheri *et al.*, 2004).



**Figure 1.5.** Schematic representation of EP receptors signaling (adapted from [www.yorku.ca/dakc/research.html](http://www.yorku.ca/dakc/research.html)).

PGE<sub>2</sub> can exert both pro-inflammatory and anti-inflammatory effects, and these actions are often produced through regulation of receptor gene expression in relevant tissues. For instance, hyperalgesia, a sign of inflammation, is mediated mainly by PGE<sub>2</sub> through EP1 receptor (Moriyama *et al.*, 2005). Other studies have also implicated EP3 receptor in the inflammatory pain response mediated by low doses of PGE<sub>2</sub> (Minami *et al.*, 2001). On the other hand and in some cases, PGE<sub>2</sub> acting through the EP2 receptor has been shown to inhibit leukocyte cytokine production, chemotaxis, and superoxide generation as well as mast cell histamine release, revealing anti-inflammatory properties of this receptor (Ying *et al.*, 2006). A recent study had demonstrated that EP4 receptor is responsible for PGE<sub>2</sub>-induced relaxation of human airway smooth muscle, showing bronchodilator effects of this receptor (Buckley *et al.*, 2011).

As opposed to many other parts of the body, the lungs represent a peculiar site for the action of PGE<sub>2</sub>. At this level, the concentration of PGE<sub>2</sub> is normally much higher than in plasma, and evidence suggest that, in this specific tissue, PGE<sub>2</sub> has a different role and its increase might be of therapeutic benefit (Vancheri *et al.*, 2004). Effectively, in the lungs PGE<sub>2</sub> has a role in limiting the immune inflammatory response as well as in controlling tissue repair processes. For instance, in bronchial asthma, PGE<sub>2</sub> prevents early and late allergen-induced bronchoconstriction, and reduces airways hyperresponsiveness and inflammation. Additionally, evidence suggest that the inhalation of PGE<sub>2</sub> can prevent the symptoms provoked by allergens. PGE<sub>2</sub> controls other important aspects of allergic inflammation by reducing eosinophil degranulation and inhibiting TxA<sub>2</sub> and cysteinil LTs (Cys-LTs) synthesis; both of them are considered potent bronchoconstrictors of asthmatic airways. Additionally, PGE<sub>2</sub> reduces AA-induced release of TxA<sub>2</sub> from human bronchial biopsies, and regulates the synthesis of LT biosynthesis (Vancheri *et al.*, 2004).

Several studies have been performed in order to demonstrate the regulatory role of PGs in inflammation, more specifically on the feedback control of COX enzymes. These investigations suggest the presence of a positive feedback of PGE<sub>2</sub> on COX-2 expression, but not in COX-1 protein

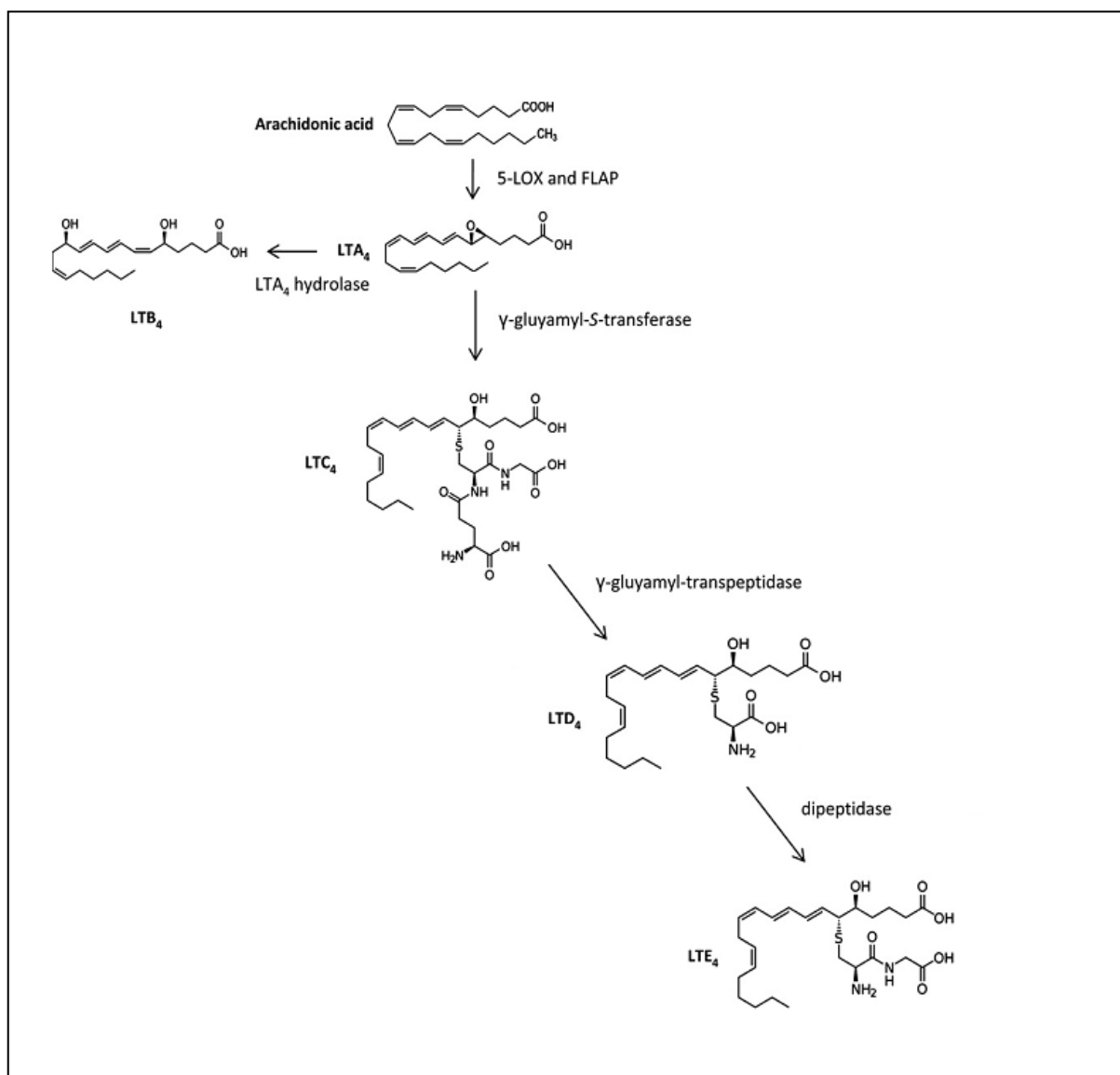
expression (Vichai *et al.*, 2005). Effectively, a deeper knowledge about the relationship between the PGs and the COX enzymes might be useful to develop new therapeutic approaches.

### 1.3.2. LOX Pathway

AA is also metabolized by LOX enzymes, found as 5-, 12-, and 15-LOX. 5-LOX plays an important role in the inflammation because it is responsible to the LT synthesis. Thus, this section will be based on this specific pathway (Picado, 2006).

The 5-LOX is responsible for the production of substances of anaphylaxis, as LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, which are potent mediators of allergic response, and LTB<sub>4</sub>, which is a powerful polymorphonuclear leukocyte chemoattractant. Upon activation, 5-LOX interacts with its processing protein, FLAP, allowing the oxygenation of AA. The product of 5-lipoxygenation of AA, LTA<sub>4</sub>, can be transformed, in some cells, into LTB<sub>4</sub> or into LTC<sub>4</sub>. The LTC<sub>4</sub> is then transported to the exterior of the cells, where is metabolized into LTD<sub>4</sub>, which can be further modified into LTE<sub>4</sub> (Figure 1.6). 5-LOX, contrary to COX enzymes, is inactive in quiescent cells but becomes enzymatically functional when the cells are activated by the increase of intracellular calcium (Stables and Gilroy, 2011).

LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> form the Cys-LTs. The Cys-LTs act through specific receptors (Cys-LTs receptor 1 and 2) of the rhodopsin class, located on the outer leaflet of the plasma membrane. Once bound to a specific receptor, is sent a signal via G-protein in the cytoplasm to increase intracellular calcium and block formation of cAMP, which alters several cellular activities. The Cys-LTs are responsible to the development of bronchoconstrictor effects, mucus secretion, and edema accumulation in airways (Gohil *et al.*, 2010; Picado, 2006). In patients with asthma or CRS who suffer from aspirin intolerance it was observed an over-expression of Cys-LTs receptor 1 (Sousa *et al.*, 2002).



**Figure 1.6.** Representation of the LT metabolism. AA is converted into LTA<sub>4</sub> by the action of 5-LOX and FLAP. LTA<sub>4</sub> can be transformed into LTB<sub>4</sub> or LTC<sub>4</sub>. LTC<sub>4</sub> is metabolized into LTD<sub>4</sub>, which is further transformed into LTE<sub>4</sub> (adapted from Stables and Gilroy, 2011).

#### 1.4. Alterations in AA Pathway

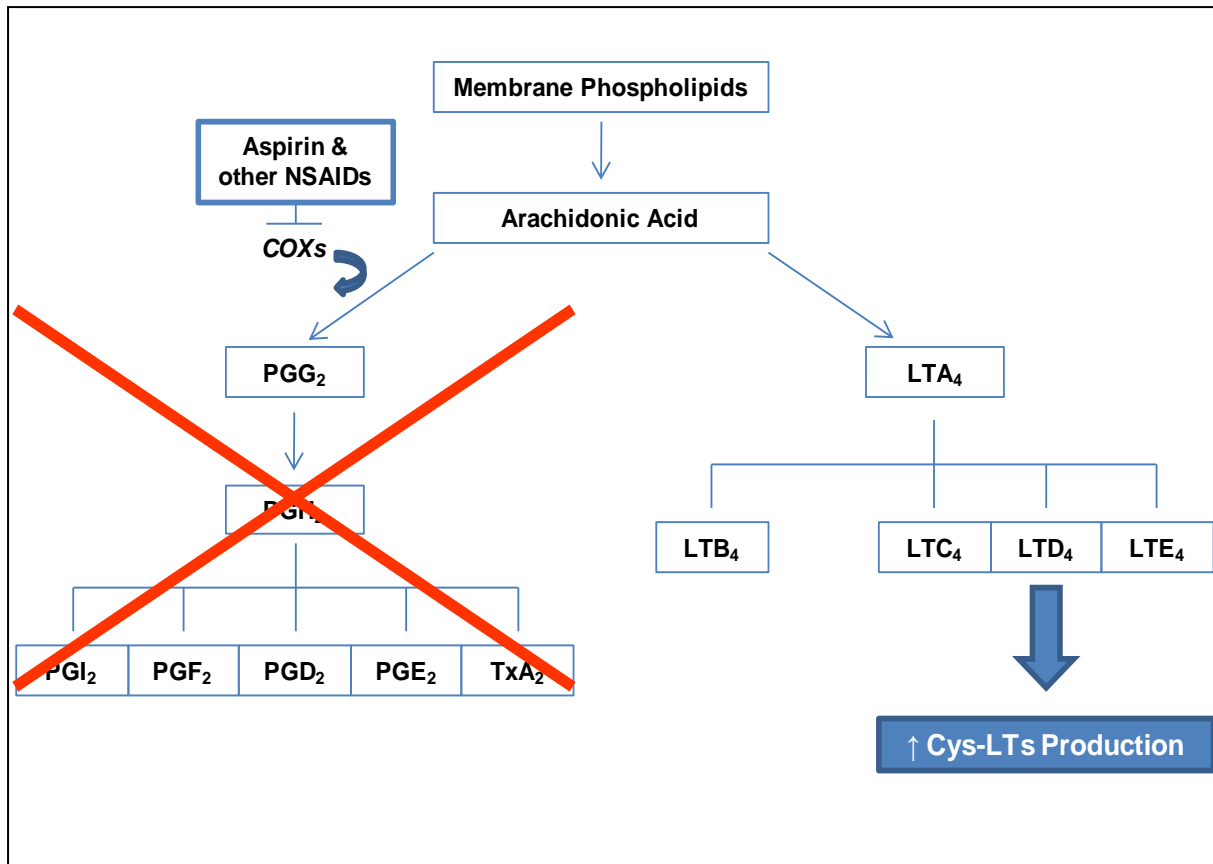
AIA is a clinical syndrome where the interplay between the two enzymatic pathways (COX and LOX) is evident and is characterized by several abnormalities in the biosynthesis of eicosanoid mediators and eicosanoid receptors. Effectively, there are several observations that indicate the presence of a complex change in the AA metabolism of NSAID-intolerant asthmatic patients. Additionally, these alterations affect practically all the pathways (Picado, 2006; Vancheri *et al.*, 2004).

The LOX pathway is more activated in asthmatic patients in basal conditions when compared with control subjects. Moreover, this pathway seems to be more activated in patients with AIA than in

tolerant patients (Picado, 2006). It has been suggested that in patients who suffer from AIA, COX inhibition by aspirin or other NSAIDs might cause shunting of AA into the 5-LOX pathway. This specific condition triggers the synthesis of the bronchoconstrictor and vasoactive Cys-LTs (LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>) by the 5-LOX/LTC<sub>4</sub> synthase pathway (Figure 1.7) (Adamjee *et al.*, 2006; Vancheri *et al.*, 2004). Several studies have demonstrated that in both upper and lower airways of patients who suffer from AIA, the 5-LOX pathway of AA is significantly up-regulated. Additionally, it was verified that the critical enzyme responsible for Cys-LT production (LTC<sub>4</sub> synthase) is much higher in the airways of patients with AIA compared with AT asthmatic patients and control subjects (Gohil *et al.*, 2010; Adamjee *et al.*, 2006; Cowburn *et al.*, 1998). Thus, the abnormal activity of the 5-LOX pathway leads to the baseline over-production of Cys-LTs in patients with AIA, which is further increased when patients are exposed to aspirin or other NSAIDs (Kowalski *et al.*, 2000; Picado *et al.*, 1992).

Several abnormalities have also been described in COX pathway of patients with AIA (Stevenson and Szczeklik, 2006; Picado, 2006). However, the role of COX enzymes in the pathogenesis of AIA is less clear, when compared with the role of LTs. Clinical studies show that inhibition of COX-1, but not COX-2, precipitates asthmatic attacks (Szczeklik and Sanak, 2006). COX-1 inhibitors accelerate depletion of PGE<sub>2</sub>, which would normally protect against the bronchoconstriction and mast-cell mediator release effects (Szczeklik and Stevenson, 2003). Additionally, expression of the COX-2 enzyme and its activity are reduced in patients with AIA (Szczeklik and Sanak, 2006). The combination of a low activity of COX-2 in aspirin-sensitive subjects with the inhibition of COX-1 by aspirin and other NSAIDs may contribute to reduced PGE<sub>2</sub> production. Effectively, numerous studies that assessed PGE<sub>2</sub> production in patients who suffer from AIA have reported a reduced production of PGE<sub>2</sub> associated with a down-regulation in the expression of both COX-1 and COX-2 in NP tissues, as well as in fibroblasts and epithelial cells derived from NPs of patients with aspirin-intolerance (Roca-Ferrer *et al.*, 2011; Yoshimura *et al.*, 2008; Pérez-Novo *et al.*, 2005; Pujols *et al.*, 2004; Picado *et al.*, 1999). On the other hand, and contrary to the results obtained in NPs, studies that used cultured fibroblasts and bronchial biopsies have not find any differences in the expression of both COX-1 and COX-2 enzymes between patients with AIA and AT or in control subjects (Pierzchalska *et al.*, 2003; Cowburn *et al.*, 1998).





**Figure 1.7.** Schematic representation of the aspirin/NSAIDs effects in patients with AIA. The image shows the shunting of AA into the 5-LOX pathway, when COX pathway is inhibited by aspirin or other NSAIDs in patients with AIA.

The expression of EP receptors also seems to be altered in patients who suffer from AIA. A study performed to measure the expression of all EP receptors in nasal biopsies from patients with and without aspirin sensitivity demonstrated a reduced number of eosinophils that express EP2 receptor in NP samples from patients with AIA, when compared with AT patients (Ying *et al.*, 2006). Moreover, it was reported that a pro-inflammatory stimulus, as interleukin (IL)-1 $\beta$ , stimulates the expression of EP2 in cultured fibroblasts from NM of control subjects, but has no effect on EP2 expression in cultured fibroblasts from NP-AIA. Since PGE<sub>2</sub> acts, in some cases, through EP2 receptor to mediate anti-inflammatory responses, the alterations verified to the EP2 receptor, as reduced expression, might be involved in the increase of the inflammatory process in the airways of patients who suffer from AIA, when compared with control subjects (Roca-Ferrer *et al.*, 2011).

## 1.5. Experimental Model

*In vitro* cell-based studies have allowed detailed investigations of the molecular mechanisms underlying the pathology of airway diseases. The fibroblasts are cells that can be used as model to investigate the mechanisms involved in the process of inflammatory airway diseases. In our

laboratory, for instance, fibroblasts are isolated from human tissue explants, obtained during surgical procedures. These samples preserve many of the *in vivo* tissue characteristics of each patient that participate in the study, for instance the cells retain the AA abnormalities reported in the patients who suffer from AIA. Effectively, previous studies that assessed the production of PGE<sub>2</sub>, the expression of COX enzymes, and the expression of all EP receptors have used cultured fibroblasts as *in vitro* model (Roca-Ferrer *et al.*, 2011).

## 1.6. Hypothesis

As previously described, several anomalies in the COX pathway in AIA have been reported, but some discrepancies were found when comparing upper and lower airways. These discrepancies might be due, at least in part, because the regulation of AA metabolism was extensively studied in upper airways but not in lower airways.

Upper and lower airways histology and physiology show many similarities and some differences, for instance smooth muscle is present in lower airways and not in upper airways. In addition, some inflammatory diseases that affect the upper airways (rhinitis and sinusitis) share some common physiopathological mechanisms with those affecting the lower airways (asthma). However, the inflammatory response in upper airways is sometimes associated with the presence of polyps, inflammatory structures never seen in the lower airways of patients who suffer from asthma and other inflammatory diseases of the lower airways. Effectively, most of the alterations of the COX pathway were verified in patients with nasal polyposis, and these alterations might be involved in the pathogenesis of nasal polyposis. Considering that, it was hypothesized that the abnormalities reported in the COX pathway are specific of NPs and not representative of the lower airways mucosa.

Additionally, there were found differences in the expression of EP receptors in nasal tissues. However, there are no studies that evaluate the expression of EP receptors in both upper and lower airways. Considering that, it was also hypothesized that differences in the expression of EP receptors also exist between upper and lower airways.

## 1.7. Objectives

### 1.7.1. General Objectives

To compare the secretion of PGE<sub>2</sub> and the expression of both COX enzymes (COX-1 and COX-2) and all EP receptors (EP1 – EP4) in upper and lower airways of control subjects, patients aspirin-tolerant who suffer from CRS with NPs, and patients aspirin-intolerant who suffer from bronchial asthma and CRS with NPs.

### **1.7.2. Specific Objectives**

1. To investigate the secretion of PGE<sub>2</sub> at baseline and after cell stimulation with a pro-inflammatory stimulus for 24 hours.
2. To analyze the expression of COX-1 at baseline and after cell stimulation with a pro-inflammatory stimulus for 24, 48, and 72 hours.
3. To study the expression of COX.2 at baseline and after cell stimulation with a pro-inflammatory stimulus for 24 hours.
4. To study the expression of COX-1 and COX-2 after cell stimulation with PGE<sub>2</sub> at different concentrations for 24 hours.
5. To analyze the expression of each EP (EP1 - EP4) receptor at baseline and after cell activation with a pro-inflammatory stimulus for 24, 48, and 72 hours.



## 2. Materials and Methods

In Table 2.1 is represented the list of all material and reagents used to carry out the experimental protocols, as well as their origin.

**Table 2.1.** List of materials and reagents.

| Materials and Reagents                                | Origin                               |
|---|--------------------------------------|
| 6-well culture plates                                 | NUNC, Wiesbaden, Germany             |
| 24-well culture plates                                | NUNC, Wiesbaden, Germany             |
| 96-well culture plates                                | NUNC, Wiesbaden, Germany             |
| 75-cm <sup>2</sup> culture flasks                     | NUNC, Wiesbaden, Germany             |
| 150-cm <sup>2</sup> culture flasks                    | NUNC, Wiesbaden, Germany             |
| 0.05% Trypsin – 0.02% Ethylenediaminetetraacetic acid | Invitrogen, United Kingdom           |
| 4',6-diamidino-2-phenylindole                         | Sigma Chemical Co., St. Louis, USA   |
| Agarose   | Conda Laboratories, Spain            |
| Amphotericin B solution (2µg/mL)                      | Sigma, St. Louis, USA                |
| Bovine Serum Albumin                                  | Sigma Chemical Co., St. Louis, USA   |
| Cell Proliferation Kit II (XTT)                       | Roche Diagnostics, Mannheim, Germany |
| • XTT labelling reagent                               |                                      |
| • Electron-coupling reagent                           |                                      |
| Complete™ Mini Protease Inhibitor Cocktail Tablets    | Roche Diagnostics, Mannheim, Germany |
| Cryoprotective media                                  | Lonza, Walkersville, USA             |
| CultureSlides®  | NUNC, Rochester, USA                 |
| Dimethyl Sulfoxide                                    | Sigma, St. Louis, USA                |
| Dulbecco's modified Eagles's media                    | Lonza, Walkersville, USA             |
| Eagle's minimal essential media                       | Lonza, Walkersville, USA             |

|  |                                       |
|--|---------------------------------------|
| Extracellular cell matrix gel  | E1270, Sigma Chemical, St. Louis, USA |
| Fetal Bovine Serum Gold (Lot A15108-1952)  | PAA Laboratories GmbH, Cölbe, Germany |
| Hepes Buffer Solution at 0.05M   | Gibco-Invitrogen, Stockholm, Sweden   |
| Neubauer chamber   | Blaubrand, Wertheim, Germany          |
| Paraformaldehyde at 4%   | Proquinorte, Spain                    |
| Pefabloc SC  | Roche Diagnostics, Mannheim, Germany  |
| Penicillin (100IU/mL) – Streptomycin (100µg/mL)  | Invitrogen, Carlsbad, USA             |
| Phosphate Buffered Saline 10X without Ca and Mg  | Lonza, Belgium                        |
| Phosphate Buffered Saline, pH = 7.4  | Sigma, St. Louis, USA                 |
| PCR <i>Mycoplasma</i> Detection Kit  | Minerva Biolabs, Germany              |
| ProLong Gold antifade reagent  | Invitrogen, USA                       |
| Protein Assay Kit  |                                       |
| <ul style="list-style-type: none"> <li>Protein Standard Solution</li> <li>Lowry Reagent</li> <li>Folin &amp; Ciocalteu's Phenol Reagent</li> <li>Working Solution</li> </ul> | Sigma, St. Louis, USA                 |
| Prostaglandin E <sub>2</sub> EIA Kit – Monoclonal  | Cayman Chemical, Ann Arbor, Mich      |
| UltraPure™ 10X<br>TRIS/Borate/Ethylenediaminetetraacetic Acid Buffer   | Invitrogen, Carlsbad, USA             |
| Restore™ Western Blot Stripping Buffer   | Thermo Scientific, Rockford, USA      |
| Super Signal® West Pico Chemiluminescent<br>Substrate  | Thermo Scientific, Rockford, USA      |
| SYBR® Safe DNA gel Stain   | Invitrogen, Oregon, USA               |
| Triton X-100   | Sigma Chemical Co., St. Louis, USA    |
| Trypan Blue Solution at 0.4%   | Sigma , St. Louis, USA                |
| Tween®20   | Sigma, St. Louis, USA                 |

|  |                                 |
|--|---------------------------------|
| NuPAGE® lithium dodecyl sulfate sample buffer 4X               | Invitrogen, Carlsbad, USA       |
| NuPAGE® sample reducing agent 10X                              | Invitrogen, Carlsbad, USA       |
| 7% TRIS-acetate gels   | Invitrogen, Carlsbad, USA       |
| Novex TRIS-acetate sodium dodecyl sulfate Running Buffer 20X   | Invitrogen, Carlsbad, USA       |
| NuPAGE® Antioxidant  | Invitrogen, Carlsbad, USA       |
| SeeBlue® Plus2 Pre-Stained Standard                            | Invitrogen, Carlsbad, USA       |
| iBlot® Anode Stack   | Invitrogen, Carlsbad, USA       |
| iBlot® Cathode Stack   | Invitrogen, Carlsbad, USA       |
| iBlot® Filter Paper  | Invitrogen, Carlsbad, USA       |
| iBlot® Disposable Sponge                                       | Invitrogen, Carlsbad, USA       |
| Prostaglandin E <sub>2</sub> , 14010                           | Cayman Chemical, Ann Arbor, USA |
| Recombinant Human Interleukin-1 $\beta$ , 201-LB               | R&D Systems, Minneapolis, USA   |
| Human $\alpha$ -smooth muscle actin monoclonal antibody, M0851 | DAKO, Glostrup, Denmark         |
| Human vimentin monoclonal antibody, V5255                      | Sigma, St. Louis, USA           |
| Pan-cytokeratin monoclonal antibody, C2562                     | Sigma, St. Louis, USA           |
| Human Cyclooxygenase-1 polyclonal antibody, sc-1752            | Santa Cruz Biotechnology, USA   |
| Human Cyclooxygenase-2 monoclonal antibody, 160112             | Cayman Chemical, Ann Arbor, USA |
| Human EP1 polyclonal antibody, 101740                          | Cayman Chemical, Ann Arbor, USA |
| Human EP2 polyclonal antibody, 101750                          | Cayman Chemical, Ann Arbor, USA |
| Human EP3 polyclonal antibody, 101769                          | Cayman Chemical, Ann Arbor, USA |
| Human EP4 polyclonal antibody, ab45863                         | Abcam, Cambridge, UK            |
| $\beta$ -actin monoclonal antibody, A2228                      | Sigma, St. Louis, USA           |

|   |   |
|---|---|
| Goat anti-rabbit IgG-HRP, sc-2004           | Santa Cruz Biotechnology, Santa Cruz, USA |
| Goat anti-mouse IgG-HRP, sc-2005            | Santa Cruz Biotechnology, Santa Cruz, USA |
| Donkey anti-goat IgG-HRP, sc-2020           | Santa Cruz Biotechnology, Santa Cruz, USA |
| Alexa Fluor 488 goat anti-mouse IgG, A11029 | Invitrogen, Carlsbad, USA                 |

## 2.1. Study Population

NM specimens were obtained from 20 non-asthmatic subjects with septal deviation, turbinate hypertrophy or both undergoing nasal corrective surgery (control-NM). All the control subjects had taken aspirin or NSAIDs at clinical dosage without any adverse reaction as asthma and/or rhinitis, urticaria, angioedema, or anaphylaxis. NM specimens were also collected from 10 asthmatic patients with CRS and AIA (NM-AIA) undergoing functional endoscopy surgery. Additionally, by means of fibrobronchoscopy during the surgery, samples of BM were collected from some of the control subjects ( $n = 5$ ) and AIA patients ( $n = 5$ ). NP specimens were collected from 18 patients with AIA (NP-AIA) and from 15 patients without asthma who tolerate aspirin (NP-AT) during the functional endoscopy surgery.

The main demographic data and clinical characteristics of the study population are shown in Table 2.2. The diagnosis of AIA was made on the basis of a clear-cut history of asthma attacks precipitated by NSAIDs and confirmed by lysine-aspirin nasal challenge as previously described (Casadevall *et al.*, 2000) in patients with an isolated episode of NSAIDs-induced asthma exacerbation. None of the control subjects had a history of nasal or sinus diseases (chronic rhinitis/rhinosinusitis) or had taken oral or intranasal corticosteroids for at least one month before surgery. None of the control subjects and patients has suffered from upper or lower airway infection during at least two weeks prior to surgery. All patients and control subjects gave an informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our Institution.



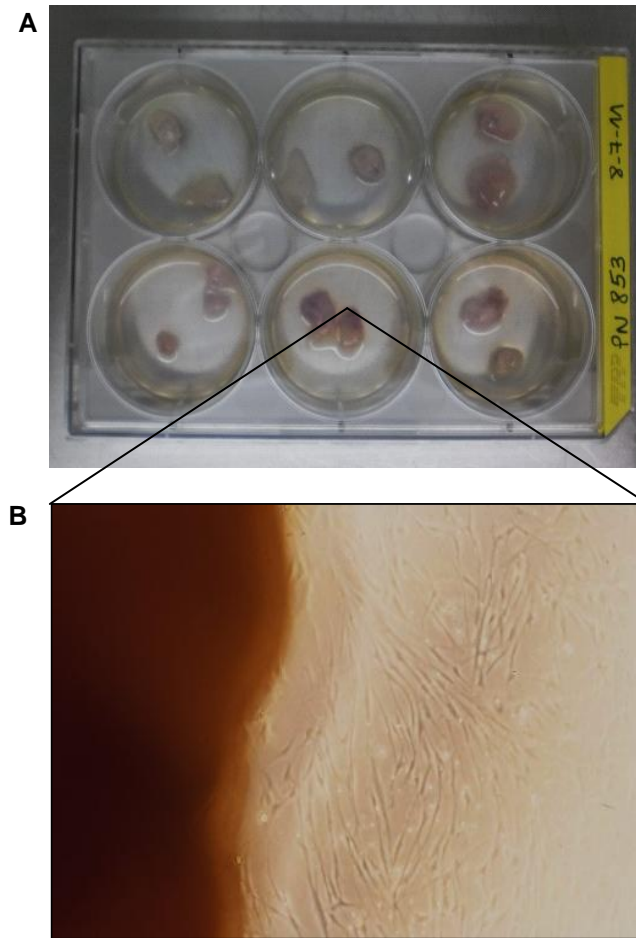
**Table 2.2.** Demographic data and clinical characteristics of the study population.

| Characteristics                   | Control-NM     | NM-AIA         | NP-AT          | NP-AIA         | Control-BM     | BM-AIA         |
|-----------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Fibroblast cultures, n            | 20             | 10             | 15             | 18             | 5              | 5              |
| Age, years (mean $\pm$ SEM)       | 44.4 $\pm$ 4.5 | 42.3 $\pm$ 3.5 | 49.1 $\pm$ 3.9 | 49.2 $\pm$ 3.4 | 32.8 $\pm$ 3.8 | 39.6 $\pm$ 2.8 |
| Female, n (%)                     | 2 (10)         | 5 (50)         | 2 (13.3)       | 11 (61.1)      | 1 (20)         | 3 (60)         |
| Asthma, n (%)                     | 0 (0)          | 10 (100)       | 0 (0)          | 18 (100)       | 0 (0)          | 5 (100)        |
| AIA, n (%)                        | 0 (0)          | 10 (100)       | 0 (0)          | 18 (100)       | 0 (0)          | 5 (100)        |
| Skin prick test positivity, n (%) | 1 (5)          | 2 (20)         | 4 (26.7)       | 6 (33.3)       | 0 (0)          | 2 (40)         |
| Intranasal corticosteroid, n (%)  | 0 (0)          | 7 (70)         | 9 (60)         | 16 (88.9)      | 3 (60)         | 5 (100)        |

AIA, aspirin-intolerant asthmatic; AT, aspirin-tolerant; BM, bronchial mucosa; NM, nasal mucosa; NP, nasal polyp

## 2.2. Tissue Handling and Cell Culture

NM and NP samples were cut into pieces of 3 X 3 mm approximately and placed in 6-well culture plates (Figure 2.1) containing 1mL of culture media (Dulbecco's modified Eagle's media (DMEM) supplemented with 10% of fetal bovine serum (FBS), 100IU/mL penicillin, 100 $\mu$ g/mL streptomycin, and 2 $\mu$ g/mL amphotericin B). The same batch of FBS was used for the whole experimental period. Since the size of the BM is very small, the tissue sections were placed on 6-well culture plates with 50 $\mu$ L of an extracellular cell matrix (ECM) gel (contains laminin as a major component, collagen type IV, heparan sulphate proteoglycan, entactin and other minor components) to fix the samples to the plastic surface. ECM gel polymerized when brought to 20 – 40°C to form a basement membrane. Then, 1mL of culture media was added to each well. Tissue pieces were kept in a 5% CO<sub>2</sub> incubator (NuAIRE US Autoflow) at 100% humidity and 37°C. Culture media was changed every 2 to 3 days.



**Figure 2.1.** Representation of fibroblast cultures. **A**, NP samples were cut into small pieces and placed in a 6-well culture plate with culture media; **B**, Microscopic view of fibroblasts growing from the NP sample piece (original magnification 4X).

Cells growth was assessed every day using an inverted optical microscope (Leica DM IRB, Leica Microsystems). When fibroblasts began to proliferate (approximately after a minimum of 3 weeks), the tissue fragments were removed and the first passage was performed. For passaging, cells were washed once with phosphate buffered saline (PBS) 1X and detached by treating with warm 0.05% trypsin / 0.02% ethylenediaminetetraacetic acid (EDTA) for five minutes at 37°C. The reaction was stopped with culture media and cells were centrifuged at 400g for 5 minutes (Hermle Z400K centrifuge, Phenix Research, NC, USA).

Cells were resuspended in culture media, seeded, and grown to 80% confluence in 75-cm<sup>2</sup> culture flasks. Then, fibroblasts were trypsinized and cultured to subconfluence in CultureSlides® and 150-cm<sup>2</sup> culture flasks to perform culture characterization and experimental protocols, respectively.

For long-term storage, subconfluent cells were trypsinized and centrifuged using the same protocol explained before. The cell suspension was resuspended in Eagle's minimal essential media (EMEM) supplemented with 20% FBS and in a solution of cryoprotective media (Basal Eagle's media with Hank's and 15% dimethyl sulfoxide (DMSO) without L-glutamine). All the process was performed

under sterile conditions. Cells were transferred to appropriate cryovials and frozen by slow cooling. After that, cells were stored under liquid nitrogen. When required, cells were thaw from liquid nitrogen and seeded with fresh culture media in order to perform new experimental protocols.

### **2.3. Polymerase Chain Reaction *Mycoplasma* Detection Kit**

When a cell-line is kept in culture, is important to test the cells for mycoplasma contamination, since mycoplasma may induce cellular changes, including chromosome aberrations, changes in metabolism and cell growth. The presence of mycoplasma was analyzed using the polymerase chain reaction (PCR)-based mycoplasma detection kit VenorGeM. It is a very sensitive test since it requires approximately only 1 to 5fg of mycoplasma deoxyribonucleic acid (DNA). The primers contained in this kit are designed for a specific region of the 16S ribonucleic acid (RNA), which is highly conserved on many *Mycoplasma* strains. The sample is mycoplasma-positive when the result is a 270 base pairs (bp) fragment. The kit provides also an internal control DNA, which can be added to the reactions. When the PCR is carried out with the internal control DNA, a successfully performed reaction is indicated by a 192 bp fragment on the agarose gel.

Samples and PCR protocol were prepared according to the instructions of the supplier. Briefly, in order to prepare the templates for this PCR assay, 100 $\mu$ L of the cell culture supernatant was collected in a sterile tube and boiled for 5 minutes, at 95°C in a thermocycler (MJ Research PTC-100, USA). After that, the tube was briefly centrifuged for 5 seconds to pellet cellular debris.

PCR reactions were carried out in a final volume of 50 $\mu$ L. Each reaction was performed by using a positive (DNA fragments of *Mycoplasma orale* genome, kit) and a negative (sterile de-ionized water) control, as well as an internal control. In Table 2.3 the instructions for *Mastermix* preparation are represented. The final volume of the *Mastermix* was 48 $\mu$ L.

**Table 2.3.** *Mastermix* for the PCR reaction.

|                                     | Volume per reaction ( $\mu\text{L}$ ) |
|-------------------------------------|---------------------------------------|
| Sterile deionized water             | 34.6                                  |
| 10X Reaction Buffer (blue cap)      | 5.0                                   |
| Primer/Nucleotide Mix (red cap)     | 5.0                                   |
| Internal Control                    | 2.0                                   |
| TAQ Polymerase (5U/ $\mu\text{L}$ ) | 0.4                                   |
| MgCl <sub>2</sub>                   | 1.0                                   |

2 $\mu\text{L}$  from the prepared cell culture were added into the reaction tube except on the tube that contains both negative and positive controls. In these tubes, 2 $\mu\text{L}$  of DNA template supplied for positive control, and 2 $\mu\text{L}$  of water for negative control were added. The amplification was performed in a thermal cycler (MiniCycler PTC-150 Thermal Cycler, MJ Research, USA) using the thermal profile shown in Table 2.4.

**Table 2.4.** Thermal profile of PCR reaction.

| Temperature ( $^{\circ}\text{C}$ ) | Time       | Cycle number |
|------------------------------------|------------|--------------|
| 94                                 | 2 minutes  | 1            |
| 94                                 | 30 seconds | 2-39         |
| 55                                 | 1 minute   |              |
| 72                                 | 30 seconds |              |
| 4 to 8                             | Unlimited  | -            |

The products of amplification were separated and visualized on a standard 1.5% agarose gel made up in 1X TRIS/Borate/EDTA buffer (0.89M Tris-HCl, 0.89M Boric Acid, 0.02M EDTA pH = 8.3) to a final volume of 1L deionized water. Syber Safe diluted 10,000X was added to the solution. The molecular weight marker used was the 100 bp DNA ladder. Each amplified PCR product (5 $\mu\text{L}$ ) was loaded and electrophoresis was carried out at 100V for 30 minutes. The bands were visualized on the charge-coupled device (CCD) Camera System LAS 3000 (Fujifilm, Tokyo, Japan).

## 2.4. Culture Characterization by Immunocytochemistry

The culture media used in the protocol is selective for fibroblast growth. However, other structural cells, such as epithelial cells, could growth in these conditions. For this reason, it was necessary to analyze the purity of the cultures. Additionally, fibroblasts may differentiate to myofibroblasts. The differential expression of structural proteins such as  $\alpha$ -smooth muscle actin (sma), vimentin, and cytokeratins has been proved to be efficient markers in differentiating myofibroblasts, fibroblasts, and epithelial cells, respectively.  $\alpha$ -sma is a contractile protein found in the cytoplasm of myofibroblasts, but not fibroblasts (Adegboyega *et al.*, 2002). Vimentin is present in myofibroblasts and fibroblasts, but not in epithelial cells. Finally, cytokeratins are found in epithelial cells, but not in fibroblasts.

To perform the experimental protocols, cells cultured in 4-well CultureSlides® were washed three times with PBS 1X (prepared from PBS 10X without calcium and magnesium), and fixed with cold paraformaldehyde at 4% for 15 minutes at room temperature. Then, cells were washed twice with PBS 1X, and incubated with a permeabilization solution that contains 0.2% of Triton X-100 in PBS 1X for 10 minutes at room temperature. The CultureSlides® were then washed twice with PBS 1X and incubated with blocking buffer, which consists to 1% bovine serum albumin (BSA) and PBS, for 1 hour in order to block non-specific bindings. Then, antibodies against  $\alpha$ -sma diluted 1:500, vimentin diluted 1:100 and pan-cytokeratin (recognizing human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19) diluted 1:200 were added for 1 hour at 37°C. Following that, the slides were washed three times in wash buffer. To undertake the detection of the primary antibody the culture slides were incubated with an appropriate fluorescent secondary antibody, Alexa Fluor 488, diluted 1:500 for 1 hour.

The cell nuclei were visualized by DNA staining using 4',6-diamidino-2-phenylindole (DAPI) diluted 1:10000. Finally, the slides with cultured cells were mounted for microscopy with a watery mounting media (ProLong Gold antifade reagent), left to solidify at least 3 hours at room temperature protected from the light. Epifluorescence microscopy (Leica Microsystems, Germany) was used to analyze the percentage of positive cells on the preparation at 200X magnification.

## 2.5. PGE<sub>2</sub> and IL-1 $\beta$ Preparation

PGE<sub>2</sub> and IL-1 $\beta$  were used in this study among the different experimental protocols in order to stimulate the cells. PGE<sub>2</sub> was dissolved in DMSO to reach a final concentration of 10<sup>-2</sup>M. To reconstitute Recombinant Human IL-1 $\beta$ , sterile PBS containing 0.1% BSA was added to the commercial vial to prepare the stock solution of 10 $\mu$ g/mL of the pro-inflammatory cytokine. The manipulation of each product was performed under sterile conditions to avoid contaminations of cell cultures. Cryovials containing PGE<sub>2</sub> at 10<sup>-2</sup>M and IL-1 $\beta$  at 10 $\mu$ g/mL were then aliquoted to avoid repeated freeze-thaw cycles and stored under sterile conditions at -80°C.

## 2.6. XTT Assay to Evaluate DMSO Effect

DMSO is a substance that at high concentrations may have cytotoxic effects on cell cultures. Since the higher PGE<sub>2</sub> concentration used in experimental protocols was 10  $\mu$ M, the maximum concentration of DMSO used to dissolve PGE<sub>2</sub> was 0.1%.

The possible toxic effect of this reagent on fibroblasts survival/viability was determined by XTT assay. Metabolic active cells reduce the tetrazolium salt (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) to orange colored compounds of formazan. The dye formed is water soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The intensity of the dye is proportional to the number of metabolic active cells. Briefly, the viability of cells was checked through microscope observation on a Neubauer Chamber by Trypan Blue exclusion. Then, cells were plated in 24-well culture plates (50 000 cells / well) with culture media. Cells were allowed to attach overnight. On the following day, cells were growth-arrested by the incubation with serum-free media (SFM) for 24 hours. Then, fibroblasts were incubated for additional 24 hours in presence or absence of 0.1% DMSO diluted in SFM. After the treatment, XTT mixture was prepared (protecting from the light and under sterile conditions) by mixing XTT labeling reagent and electron-coupling reagent according to the instructions of the supplier. The cells were washed with sterile PBS 1X and the XTT mixture previously prepared was added to each well. The culture plates were incubated at 37°C, and the absorbance was measured after the incubation period (2 and 4 hours) at 490 nanometres (nm) using a microplate spectrophotometer (Thermo Multiskan EX).

## 2.7. Experimental Protocols

It has been reported that COX expression, in response to exogenous stimulus, is more robust in quiescent fibroblasts than in proliferating cells. Primary fibroblasts cultured in SFM for 24 – 48h are morphologically and biochemically different from those cultured in 10 – 20% serum. These cells are considered quiescent (Wu, 2007). Primary human fibroblasts continue to exhibit high metabolic rates when induced into quiescence via serum withdrawal. Additionally, most studies have carried out the experimental protocols from subconfluent cell cultures. Considering these evidence, to perform the various experimental protocols, and in order to compare the results of this study with previous published data, the cells were started when cultures were subconfluent and after incubation with SFM for 24 hours. For that step, cells were washed once with warm PBS 1X and fresh SFM was added to the culture flasks before each experimental protocol.

### 2.7.1. Basal Secretion of PGE<sub>2</sub> and Basal Expression of COX Enzymes and EP Receptors

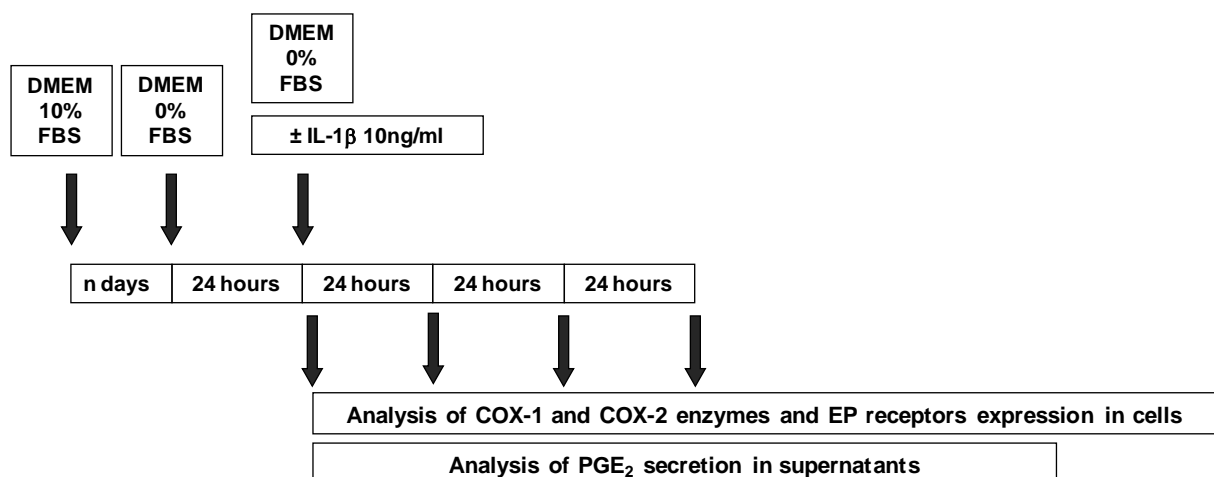
After incubation with SFM for 24 hours, culture supernatants were collected and used to analyze basal secretion of PGE<sub>2</sub> by means of Enzyme-Linked ImmunoSorbent Assay (ELISA). In order to analyze the basal expression of COX enzymes and EP receptors, cell lysates were obtained and used to study the basal expression of COX-1, COX-2, EP1, EP2, EP3 and EP4 protein by means of immunoblot analysis (Figure 2.2).

### 2.7.2. Effect of IL-1 $\beta$ on PGE<sub>2</sub> Secretion and COX Enzymes and EP Receptors Expression

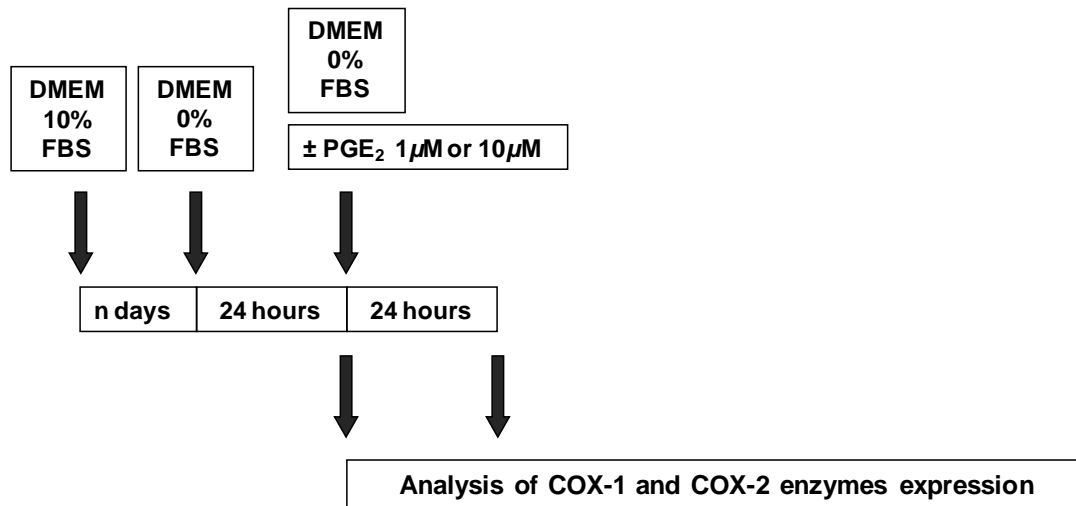
To evaluate the effect of IL-1 $\beta$  on secretion of PGE<sub>2</sub> and expression of COX enzymes and EP receptors cells were incubated with fresh SFM in the presence of 10ng/mL IL-1 $\beta$ , for different times. Supernatants and cells incubated for 24 hours with IL-1 $\beta$  were collected to analyze PGE<sub>2</sub> secretion by means of ELISA and COX-2 protein expression by means of immunoblot, respectively (Figure 2.2). To analyze COX-1, EP1, EP2, EP3, and EP4 expression, by means of immunoblot, the cells were stimulated for 72 hours with IL-1 $\beta$ , and cell lysates were collected to each 24 hours (Figure 2.2).

### 2.7.3. Effect of PGE<sub>2</sub> on the Expression of COX Enzymes

To evaluate the effect of PGE<sub>2</sub> on the expression of COX enzymes, fibroblasts were incubated with fresh SFM in the presence of 1 or 10 $\mu$ M PGE<sub>2</sub> during 24 hours and cell lysates were used to analyze the expression of COX-1 and COX-2 by means of immunoblot (Figure 2.3).



**Figure 2.2.** Design of the experimental protocols performed to analyze COX-1 and COX-2 enzymes and EP receptors expression, and PGE<sub>2</sub> secretion.



**Figure 2.3.** Design of the experimental protocols performed to analyze the expression of both COX-1 and COX-2 enzymes.

## 2.8. ELISA for PGE<sub>2</sub>

To measure the secretion of PGE<sub>2</sub>, culture supernatants were collected and centrifuged at 400g for 10 minutes at 4°C, in a refrigerated centrifuge (Eppendorf, Centrifuge 5417 R), sterilized through 0.22- $\mu$ m filters, and stored at -80°C until the measurement.

The concentration of PGE<sub>2</sub> was measured using a specific kit (Prostaglandin E2 EIA Kit – Monoclonal) following the manufacturer's instructions. The assay is based on the principle that *in vivo* PGE<sub>2</sub> is rapidly converted into an inactive metabolite (13,14-dihydro-15-keto PGE<sub>2</sub>) by the PG 15 dehydrogenase pathway (Granström *et al.*, 1980; Hamberg and Samuelsson, 1971).

Briefly, the content of one vial of EIA Buffer Concentrate was diluted with 90mL double-distilled water (ddH<sub>2</sub>O). Care was taken to rinse the vial to remove any salts that may have precipitated. The content of a 2.5mL vial of Wash Buffer Concentrate was diluted to a total volume of 1L with ddH<sub>2</sub>O and then 0.5mL Tween®20 was added. The content of the PGE<sub>2</sub> standard was reconstituted with 1mL of EIA Buffer and stored at 4°C. The standard was prepared through serial dilution from 7,8pg/mL to 1000pg/mL. The PGE<sub>2</sub> acetylcholine esterase tracer and the PGE<sub>2</sub> monoclonal antibody were both reconstituted with 6mL EIA Buffer. To perform the assay, the reagents and 50 $\mu$ L of samples, in duplicate, were added to the plate according to the manufacturer's instructions. The plate was covered and incubated for 18 hours at 4 °C. When the plate was ready to develop, Ellman's Reagent was reconstituted in 20mL ddH<sub>2</sub>O. The wells of the plate were rinsed 5 times with Wash Buffer before adding 200 $\mu$ L Ellman's Reagent to each well. The plate was re-covered, to protect it from the light, placed in a dark box and incubated at room temperature for 60 – 90 minutes on an orbital shaker. Once incubated, the plate was read at 405 nm using a microplate spectrophotometer (Thermo Multiskan EX).



## 2.9. Immunoblot Analysis

### 2.9.1. Preparation of Protein Lysates from Cells

Following experimental treatments, protein lysates were obtained. Cells were washed three times with ice-cold PBS 1X and scrapped in PBS 1X to detach the cells from the surface of the culture flasks. Cells were then collected into an appropriate tube and centrifuged at 1500g for 5 minutes at 4°C (Eppendorf, Centrifuge 5417 R). The cells were resuspended in 0.4mL of ice cold lysis buffer that contains a complete protease inhibitor cocktail tablet in 50mL of 0.05mol/L Hepes buffer solution, 0.05% v/v Triton X-100 and 625 $\mu$ mol/L phenylmethylsulfonyl fluoride (Pefabloc). The lysates were maintained for 20 minutes on ice and then collected and frozen at -80°C for further utilization.

When required, the cell suspension was thawed and thereafter sonicated at 35% amplitude in ice, twice, for 15 seconds, in a sonifier (Branson Sonifier 250, Danbury, CT, USA) and centrifuged at 12,000g for 10 minutes, at 4°C. Supernatant containing protein lysates were then collected and stored at -80°C.

### 2.9.2. Protein Concentration Determination

To ensure an equal loading in the protocol of immunoblot assay, the amount of protein in cell lysates was quantified, using a Protein Assay Kit based on the Lowry method. The kit measures the total protein using known BSA concentrations as standard. Briefly, the standards were prepared by the dilution of *Protein Standard Solution* in deionized water to a final volume of 250 $\mu$ L, as shown in Table 2.5. The blank was prepared adding 250 $\mu$ L of water. Samples were analyzed at 1:50 dilution in water and the Lowry Reagent was added and mixed in each tube and left for 20 minutes. After that, the Folin & Ciocalteu's Phenol Reagent Working Solution was added and mixed in each tube to allow colour to develop (30 minutes after). The content of each tube was transferred to a 96-well plate and all samples were quantified in duplicates and averages were used to perform the quantification. The absorbance was measured at 630 nm using a microplate spectrophotometer (Thermo Multiskan EX).

**Table 2.5.** Standards preparation for calibration curve.

| Protein Concentration<br>( $\mu\text{g/mL}$ ) | Protein Standard Solution<br>( $\mu\text{L}$ ) | Water ( $\mu\text{L}$ ) |
|---|--|-------------------------|
| 0   | 0  | 250                     |
| 50  | 31.2   | 218.7                   |
| 100   | 62.5   | 187.5                   |
| 200   | 125  | 125                     |
| 300   | 187.5  | 62.5                    |
| 400   | 250  | 0                       |

### 2.9.3. Protein Electrophoresis

Under denaturing conditions, protein samples can be separated according to their size on acrylamide gels. Since different proteins with similar molecular weights may migrate differentially due to their differences in secondary, tertiary or quaternary structure, sodium dodecyl sulfate (SDS), an anionic detergent, is used to reduce proteins to their primary structure, giving nearly a uniform negative charge along the length of the polypeptide. The voltage applied, allows the migration of anions and the proteins are fractionated by size.

To undertake the electrophoresis, the NuPAGE system was used. Basically, 15 $\mu\text{g}$  of protein extract were denatured in the presence of a loading buffer 1:4 ratio (NuPAGE lithium dodecyl sulfate (LDS) sample buffer) and NuPAGE sample reducing agent 1:10 ratio at 70°C for 10 minutes in a thermocycler (MJ Research PTC-100, USA). Samples were loaded in 7% TRIS-acetate gels (1.5mm x 10 well) in SDS Running Buffer (Novex TRIS-Acetate SDS Running Buffer 20X), set in a vertical electrophoresis chamber, XCell Sure Lock Mini-Cell (Invitrogen). To maintain proteins in a reduced state, during protein gel electrophoresis, 500 $\mu\text{L}$  of an antioxidant (NuPAGE) was added to the running buffer. A molecular weight marker (SeeBlue Plus2 Pre-Stained Standard) was loaded on the gel as a size reference. This marker provides an easier band identification, since it indicates the molecular weight in kilodalton (kDa) of the proteins and allows a quickly evaluation of the transfer efficiency. Proteins were separated at 125V for 90 minutes using an external power source, BioRad Power Pac 1000 (BioRad Laboratories Ltd., Hemel Hemstead, UK), until the dye had run to the bottom of the gel.

#### **2.9.4. Blotting**

The proteins were transferred using a dry blotting system (iBlot® Dry Blotting System, Invitrogen). This system blots proteins from polyacrylamide gels in 7 minutes and any additional buffers or external power supply are needed. The iBlot uses disposable stacks and the bottom stack includes an integrated 0.2µm nitrocellulose membrane. The order of layers in the transfer system was from bottom to top: anode stack with a nitrocellulose membrane incorporated, acrylamide gel, filter paper, cathode stack and disposable sponge.

#### **2.9.5. Blocking**

After transfer and in order to block nonspecific binding sites the membranes were placed in a blocking buffer that contains 5% nonfat dry milk and 0.1% Tween®20 in 10nmol/L PBS for 1 hour at room temperature. This and further steps were performed carefully with gentle shaking in an orbital shaker (Stuart Scientific SO3 Orbital Shaker, UK).

#### **2.9.6. Antibodies Incubation**

Membranes were incubated with the primary antibody against COX-1, COX-2, EP1, EP2, EP3 or EP4 diluted 1:1000 in blocking buffer overnight at 4°C. Membranes were washed four times in an orbital shaker with wash buffer (0.5% Tween®20 in 10nmol/L PBS) and incubated with specific horseradish peroxidase-labeled secondary antibodies: goat anti-rabbit (EP1, EP2 and EP3), goat anti-mouse (COX-2), or donkey anti-goat (COX-1 and EP4) diluted 1:3000 in blocking buffer at room temperature for 2 hours. Then, the membranes were washed four times in wash buffer.

For β-actin protein detection, membranes were incubated with a monoclonal anti- β-actin antibody diluted 1:10000 in blocking buffer, overnight at 4°C, washed four times with wash buffer and incubated with the appropriate peroxidase-conjugated goat anti-mouse diluted 1:10000 in blocking buffer at room temperature for 2 hours.

#### **2.9.7. Detection System**

Immunoreactive bands were visualized by using a chemiluminescent method, which emits light and allows the visualization of the proteins on the membranes. To perform this step the membranes were incubated for 5 minutes with the commercial Supersignal West Pico Chemiluminescent Substrate and the bands were detected with the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band

intensities were quantified with Fujifilm Image Gauge v.4.0 Software and normalized to intensities of  $\beta$ -actin and assessed from the same samples.

### **2.9.8. Stripping of the Membranes**

Membranes were stripped to remove previously bound antibodies. This step enables detection of other proteins in the same membrane. Briefly, the membranes were washed to remove chemiluminescent substrate and incubated for 15 minutes with the commercial Restore Western Blot Stripping Buffer, at room temperature in an orbital shaker and protected from the light. After this time, the membranes were washed in wash buffer and blocked for 1 hour prior the addition of the first antibody.

### **2.10. Statistical Analysis**

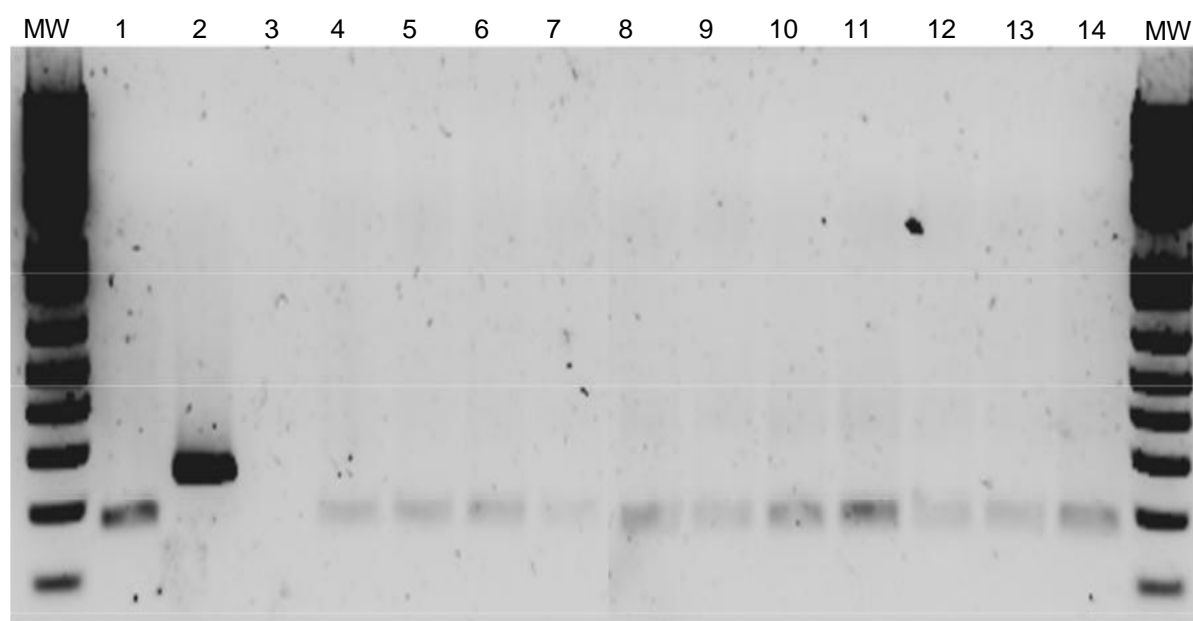
In order to perform the statistical analysis of the results, the statistical software SPSS 18.0 was used. Immunofluorescence data are expressed as medians (25<sup>th</sup> – 75<sup>th</sup> interquartiles) of positive-cell percentage among total cells. ELISA results are shown as medians (25<sup>th</sup> – 75<sup>th</sup> interquartiles) of picograms of PG per micrograms of total proteins contained in each flask. Immunoblot results are expressed as medians (25<sup>th</sup> – 75<sup>th</sup> percentiles) of band intensities normalized for  $\beta$ -actin. The non-parametric statistical Mann-Whitney *U* test was used for between-group comparisons, and the Wilcoxon rank test was used for paired comparisons after confirming differences with the Friedman test. Statistical significance was set at a p value of less than 0.05. Graphic representation of the results was performed using the scientific 2D graphing and statistics software GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, California, USA).

### 3. Results

#### 3.1. *Mycoplasma* PCR Test

The PCR reaction assay was performed in order to exclude mycoplasma contaminations of cell cultures, to guarantee the quality and purity of samples used in the protocols. The weak band at 192 bp visualized in lanes 4 to 14 that include the cell culture samples, corresponds to the internal PCR amplification control, which indicates an effective PCR process, and discards contaminations by mycoplasma. On the other hand, the strong band at 270 bp also presented in the agarose gel and observed in lane 2 corresponds to the positive control and demonstrates an effective amplification of the mycoplasma product by the PCR process. Furthermore, in order to confirm the reliability of the procedure, the negative controls were performed with and without an internal PCR amplification control, observed in the lanes 1 and 3, respectively. None of cultures used in experimental protocols were found to be positive for mycoplasma.

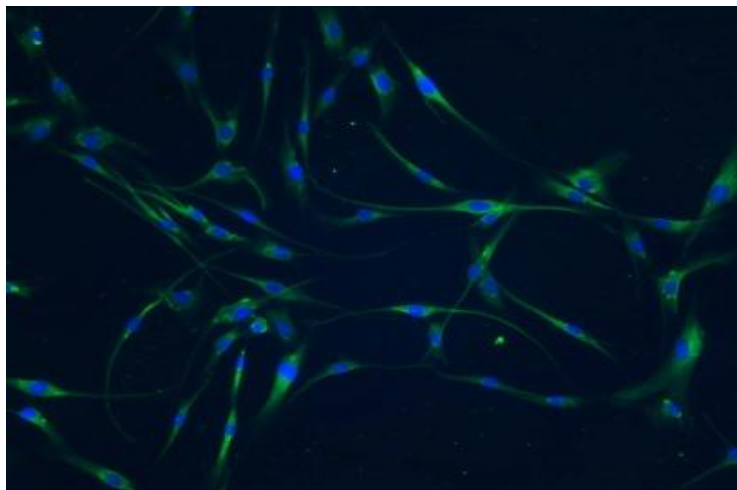
The Figure 3.1 shows an example of mycoplasma analysis.



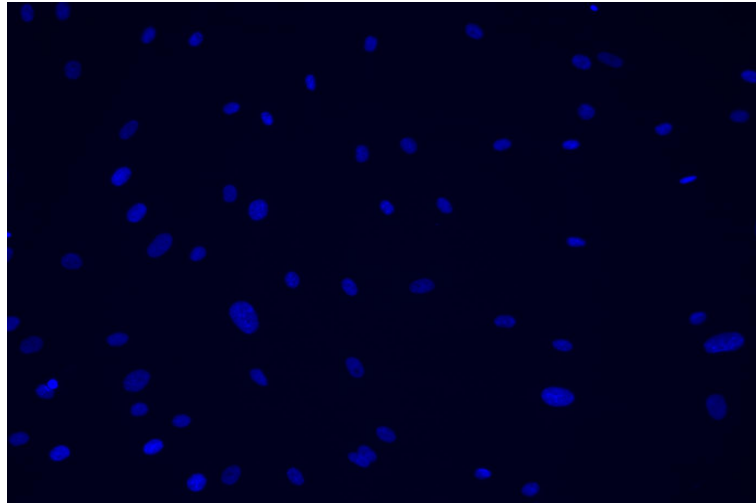
**Figure 3.1.** Representative agarose gel obtained from the DNA screening of mycoplasma by PCR. The presence of mycoplasma contamination produces a fragment of 270bp and the internal control generates a band of 192 bp. The PCR products were separated on a 1.5% agarose gel containing 10,000X dilution of Syber Safe and visualized with CCD Camera System LAS 3000. **MW:** molecular weight (standard 100 bp DNA ladder); **1:** negative control (sterile de-ionized water) with internal PCR amplification control (192 bp); **2:** positive control (DNA fragments of *Mycoplasma orale* genome, 270 bp); **3:** negative control without internal PCR amplification control; **4-14:** samples from fibroblast cultures with internal PCR amplification control (192 bp).

### 3.2. Culture Characterization

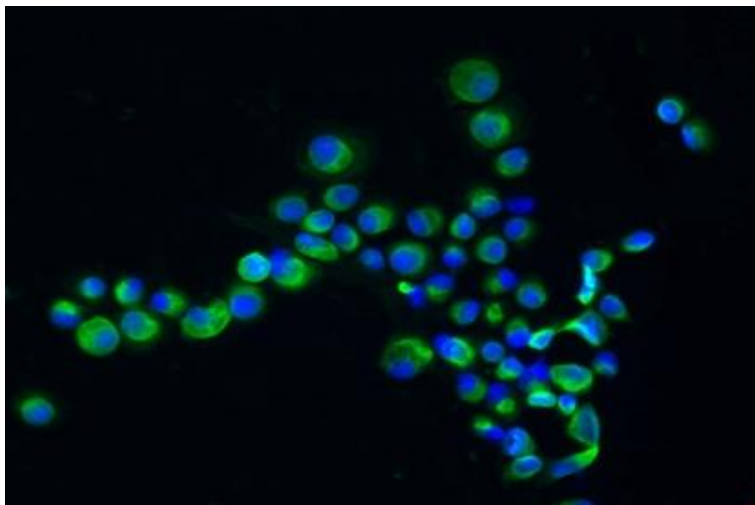
In order to test the purity of fibroblast cultures, the immunocytochemistry technique was performed. The representative images obtained from immunofluorescence analysis are presented in Figures 3.2, 3.3, 3.4. and 3.5. Cultures of control-NM, NM-AIA, NP-AT, NP-AIA, control-BM, and BM-AIA cells incubated with culture media for 24 hours did not contain cytokeratin-positive cells (Figure 3.3), whereas all cells were positive for vimentin (Figure 3.2). Myofibroblasts were also present in the cell cultures, since there were cells positive to  $\alpha$ -sma (Figure 3.5). However, no significant differences were observed in the myofibroblast percentage in the different cell cultures (control-NM = 1.6%; 1.3-3.3, NM-AIA = 3.5%; 2.0-3.6, NP-AT = 4.7%; 2.4-8.7, NP-AIA = 4.5%; 2.2-5.7, control-BM = 1.5%; 1.3-3.0, BM-AIA = 2.1%; 1.9-2.7).



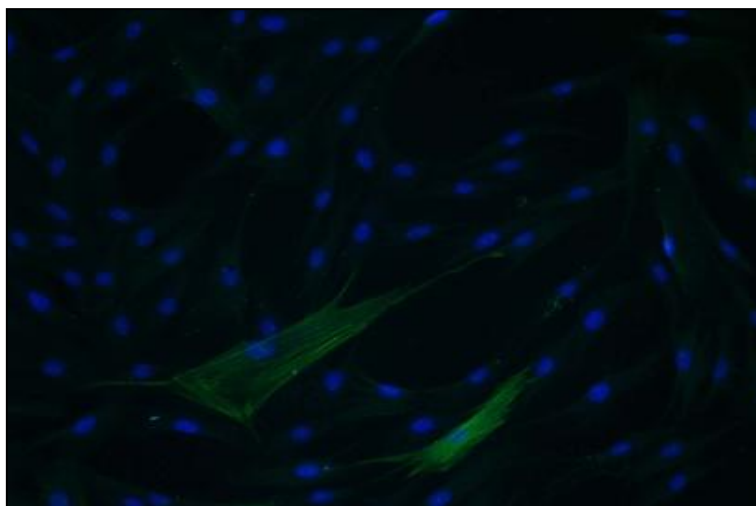
**Figure 3.2.** Representative image from immunofluorescence analysis of quiescent control-NM fibroblasts, indicating the presence of vimentin fibers. The presence of vimentin fibers (green fibers) in the cytoplasm of quiescent cells, confirms the fibroblast phenotype of cells in culture. The cell nuclei were visualized by DNA staining using DAPI diluted 1:10000 and epifluorescence microscopy was performed to analyze the positive cells on preparation (original magnification 200X).



**Figure 3.3.** Representative image from immunofluorescence analysis of quiescent control-NM fibroblasts, indicating the total absence of cyokeratins. The total absence of immunofluorescent staining for cyokeratins demonstrates de lack of epithelial cells. The cell nuclei were visualized by DNA staining using DAPI diluted 1:10000 and epifluorescence microscopy was performed to analyze the positive cells on preparation (original magnification 200X).



**Figure 3.4.** Representative image from immunofluorescence analysis of NM epithelial cells, indicating the presence of cyokeratins. The presence of immunoflorescent staining for cyokeratins confirms the epithelial phenotype of cells in culture (positive control). The cell nuclei were visualized by DNA staining using DAPI diluted 1:10000 and epifluorescence microscopy was performed to analyze the positive cells on preparation (original magnification 200X).



**Figure 3.5.** Representative image from immunofluorescence analysis of quiescent control-NM fibroblasts, indicating the presence of  $\alpha$ -sma. The detection of  $\alpha$ -sma confirms the presence of myofibroblasts in the cell cultures. The cell nuclei were visualized by DNA staining using DAPI diluted 1:10000 and epifluorescence microscopy was performed to analyze the positive cells on preparation (original magnification 200X).

### 3.3. DMSO Effect on Fibroblast Cultures

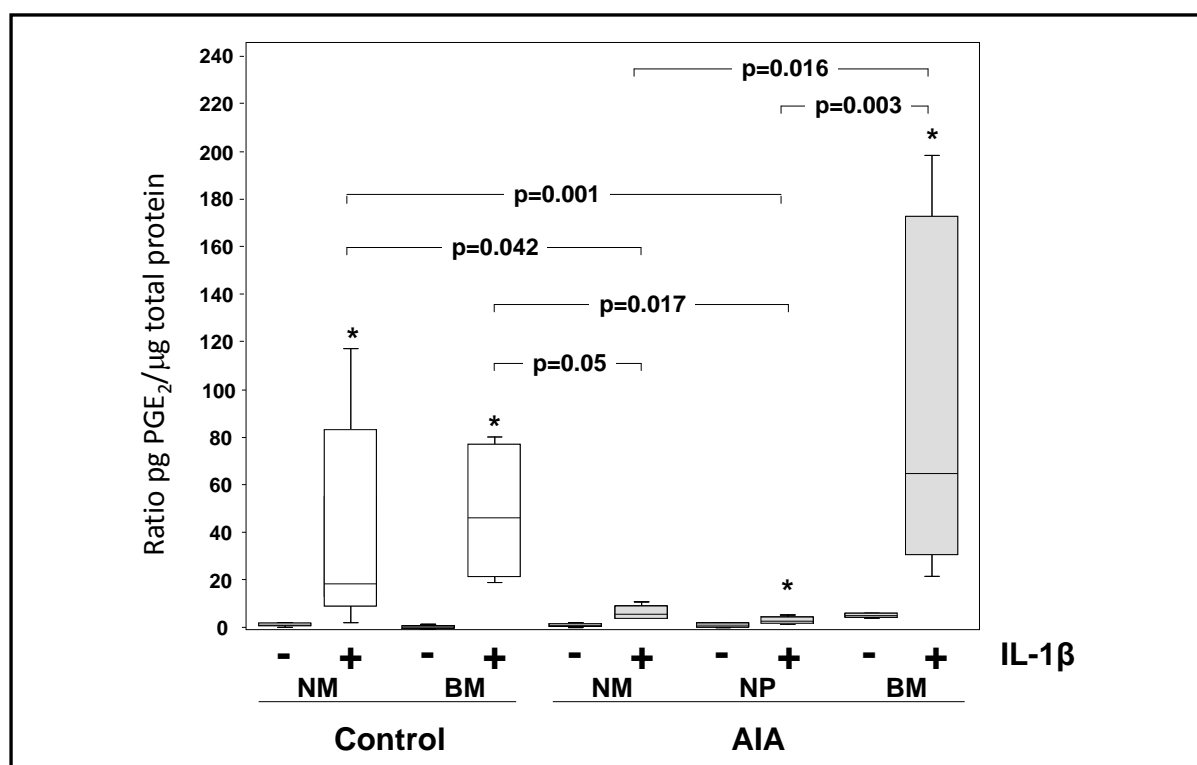
The results obtained from the XTT assay demonstrated that 0.1% DMSO (maximum concentration used to dissolve  $\text{PGE}_2$ ) did not cause any significant effect on fibroblasts viability after 24 hours of incubation with the reagent, when compared with control media (non-stimulated cells).

### 3.4. $\text{PGE}_2$ Secretion

The quiescent fibroblasts were incubated with culture media in the presence or absence of 10ng/mL IL-1 $\beta$  for 24 hours, and  $\text{PGE}_2$  secretion was analyzed in culture supernatants by means of ELISA.

At baseline, the secretion of  $\text{PGE}_2$  was low and no statistical differences were found in the secretion of  $\text{PGE}_2$  when the different fibroblast cultures were compared. However, the incubation with IL-1 $\beta$  for 24 hours significantly increased the ratio of  $\text{PGE}_2$ /total protein in control-NM, NP-AIA, control-BM, and BM-AIA, with no effect in NM-AIA. There were no differences in the IL-1 $\beta$ -induced secretion of  $\text{PGE}_2$  among fibroblasts from control-NM, control-BM, and AIA-BM. However, the ratio of  $\text{PGE}_2$ /total protein was significantly lower in cells from NP-AIA and NM-AIA compared to control-NM, control-BM and BM-AIA cells (Figure 3.6).





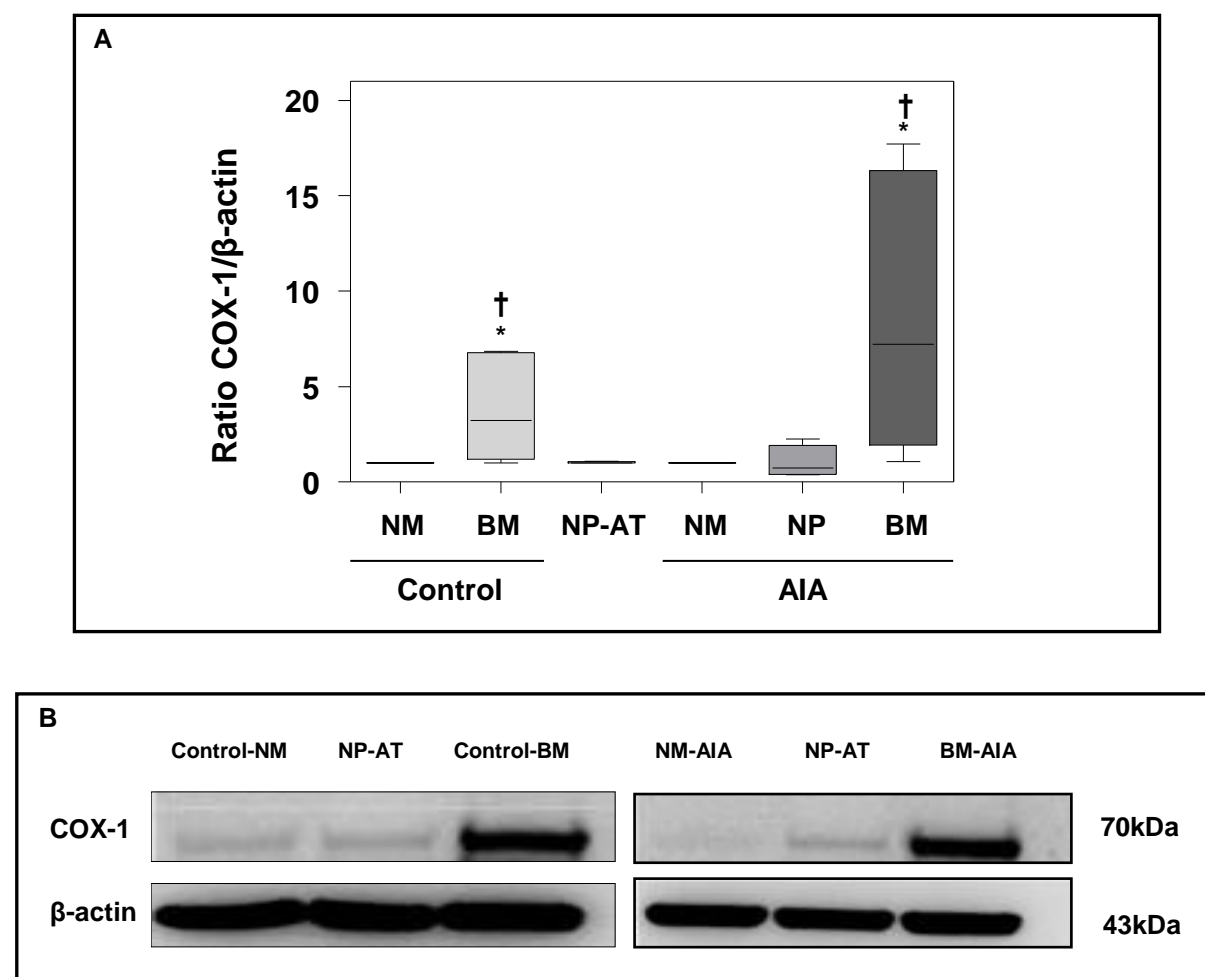
**Figure 3.6.** Effect of IL-1 $\beta$  on PGE<sub>2</sub> protein secretion. Quiescent fibroblasts from control-NM (n = 7), control-BM (n = 5), NM-AIA (n = 4), NP-AIA (n = 7), and BM-AIA (n = 3) were incubated with or without 10ng/mL IL-1 $\beta$  for 24 hours. The secretion of PGE<sub>2</sub> was measured by means of ELISA. The figure shows the significant differences in the secretion of PGE<sub>2</sub> between the different tissues and after incubation with IL-1 $\beta$ . \*  $p < 0.05$  compared with control media.

### 3.5. COX Expression

#### 3.5.1. Basal Expression of COX Enzymes

The basal expression of COX-1 protein was measured in quiescent fibroblasts incubated with control media for 24 hours. By means of immunoblot analysis, the results demonstrated that COX-1 protein expression was not different in upper airways (control-NM, NM-AIA, NP-AT, and NP-AIA). Similarly, in lower airways, no differences were found when fibroblasts from control-BM and BM-AIA were compared. However, the comparison between upper and lower airways shows that in fibroblasts from BM of control subjects, the COX-1 protein expression was higher compared to the respective control-NM. Additionally, in patients who suffer from AIA, COX-1 protein expression was higher in fibroblasts from BM when compared with the respective NM and NP (Figure 3.7).

The basal expression of COX-2 was also analyzed in all fibroblast cultures. However, the expression of the protein was not detected in any fibroblast cultures, probably because COX-2 is an inducible enzyme only induced under inflammatory conditions.



**Figure 3.7.** COX-1 basal expression on cultured fibroblasts. Quiescent fibroblasts from control-NM (n = 3), control-BM (n = 5), NP-AT (n = 3), NM-AIA (n = 4), NP-AIA (n = 4), and BM-AIA (n = 5) were incubated for 24 hours with SFM. **A**, COX-1 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblots of COX-1 and corresponding β-actin. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with both control-NM and NM-AIA (COX-1/ β-actin ratio = 1). †  $p < 0.05$  compared with both NP-AT and NP-AIA.

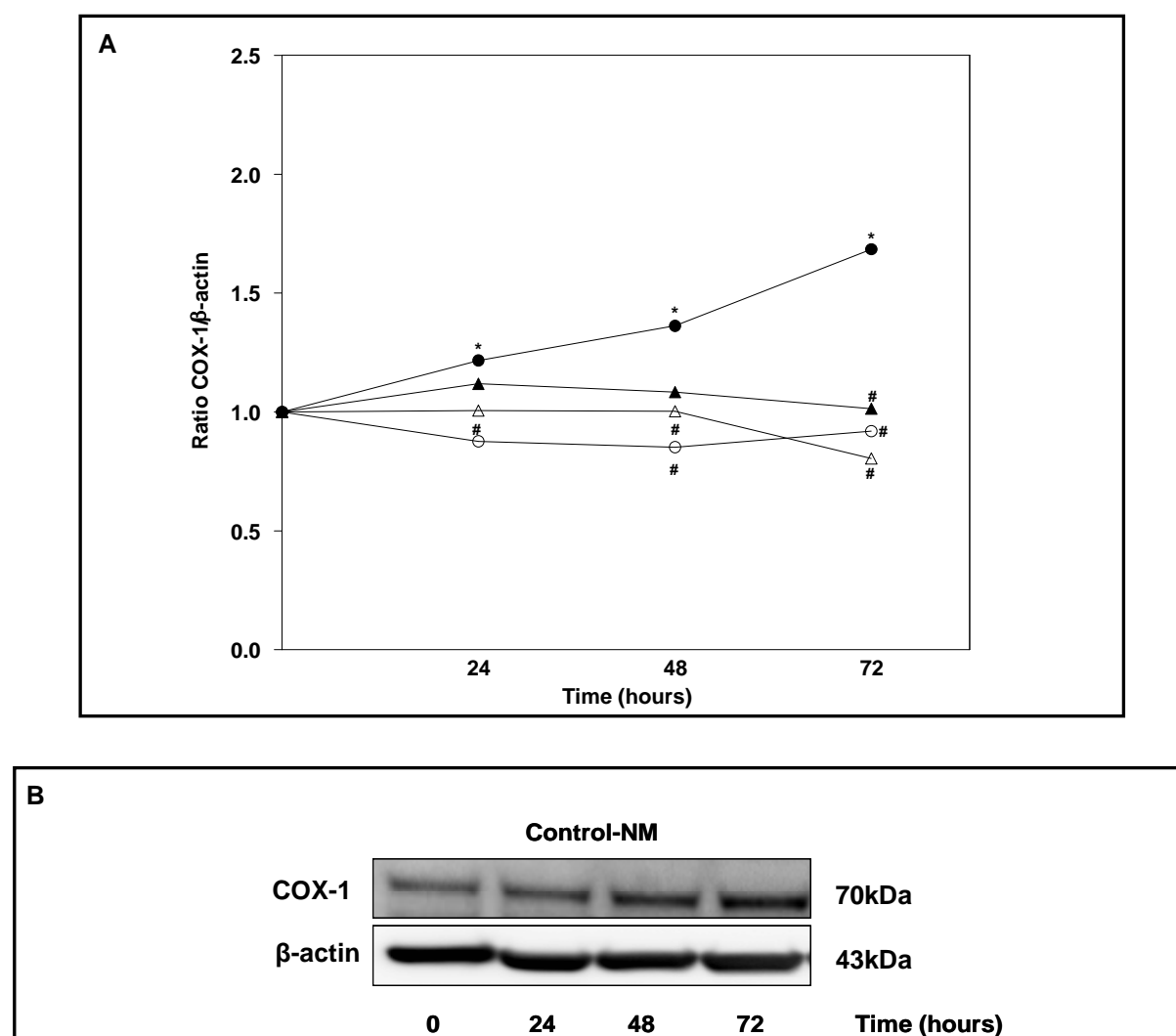
### 3.5.2. Effect of IL-1β on COX Expression

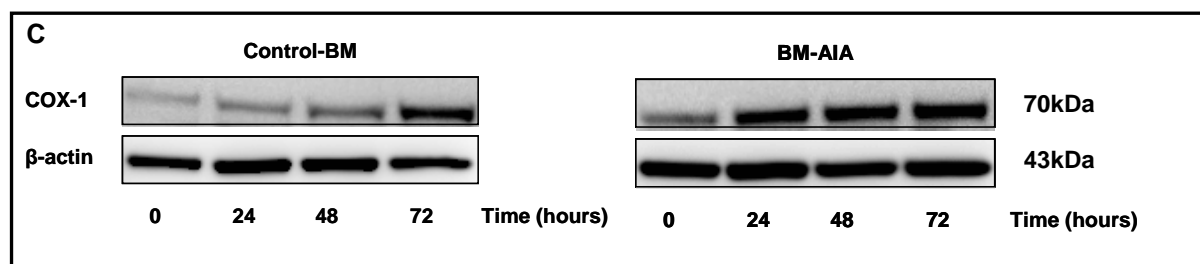
COX-1 is considered normally a non-inducible enzyme. Although, increases in its expression has been found under inflammatory conditions (Roca-Ferrer *et al.*, 2011). Thus, in order to evaluate the effect of 10ng/mL IL-1β on COX-1 protein expression, quiescent fibroblasts were incubated with the pro-inflammatory cytokine and COX-1 expression was measured at 24, 48, and 72 hours.

In control-NM fibroblasts, IL-1 $\beta$  at 10ng/mL significantly induced COX-1 protein expression at 24, 48, and 72 hours, compared with cells incubated with culture media. On the other hand, IL-1 $\beta$  had no effect on COX-1 protein expression in fibroblasts from NP-AT, NM-AIA, and NP-AIA tissues, when compared with the respective cells incubated with culture media (Figure 3.8A and B).

Additionally, compared with IL-1 $\beta$  treated control-NM fibroblasts, expression of COX-1 was significantly lower in fibroblasts from NM-AIA, NP-AIA, and NP-AT at 24 hours (NP-AIA = 1.01; 0.87-1.14), 48 hours (NM-AIA = 0.85; 0.58-1.02 and NP-AIA = 1.00; 0.87-1.14), and 72 hours (NM-AIA = 0.92; 0.68-1.14, NP-AT = 1.01; 0.76-1.54, and NP-AIA = 0.80; 0.64-0.98).

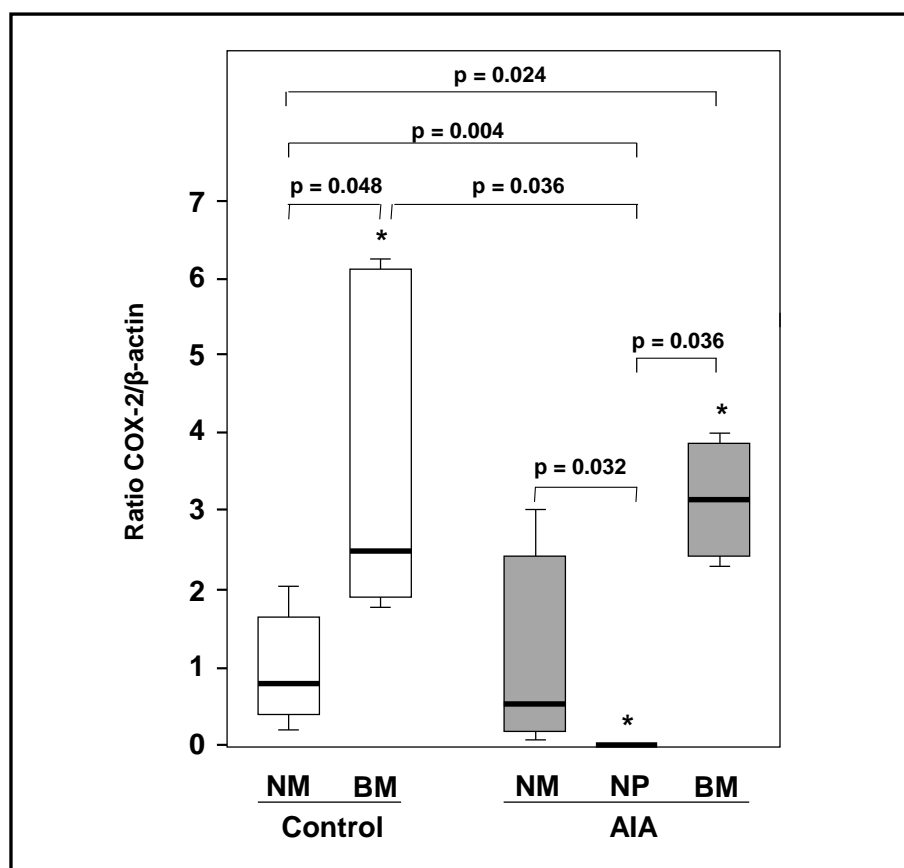
When COX-1 protein expression was analyzed in bronchial fibroblasts, the results were unclear. In fibroblasts from both BM (control and AIA), the results suggest an increase on COX-1 protein expression after stimulation with IL-1 $\beta$  at 10ng/mL (Figure 3.8C) over the time, but no differences were found in these tissues when statistical analysis was performed. In this case the variability of the results and the reduced number of samples (n = 3) available to undertake the experimental protocols failed to demonstrate any differences in the expression levels of COX-1 protein in fibroblasts from both control-BM and BM-AIA.





**Figure 3.8.** Effect of IL-1 $\beta$  on COX-1 expression. Fibroblasts from control-NM (n = 9), NP-AT (n = 9), NM-AIA (n = 6), and NP-AIA (n = 10) were incubated with 10ng/mL IL-1 $\beta$  for 72 hours. **A**, COX-1 protein expression in quiescent fibroblasts from control-NM (black circle), NM-AIA (white circle), NP-AT (black triangles), NP-AIA (white triangles) was analyzed by means of immunoblotting. **B**, Representative immunoblot of control-NM, showing the time-dependent increase of COX-1 protein expression. **C**, Representative immunoblot of both control-BM and BM-AIA, showing the slight time-dependent increase in COX-1 protein expression. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control media (COX-1/ $\beta$ -actin ratio = 1). #  $p < 0.05$  compared with control-NM fibroblasts incubated with IL-1 $\beta$ .

In order to evaluate the effect of 10ng/mL IL-1 $\beta$  on COX-2 expression, fibroblasts were incubated with the pro-inflammatory cytokine and the COX-2 expression was measured at 24 hours by means of immunoblot. Compared with control-NM incubated with IL-1 $\beta$ , the expression of COX-2 was significantly increased in fibroblasts from control-BM (2.48; 1.97-6.26) and BM-AIA (3.15; 2.45-3.96) and significantly decreased in fibroblasts from NP-AIA (0.01; 0.00-0.03). The lowest ratio COX-2/ $\beta$ -actin was verified in fibroblasts from NP-AIA, since the values were significantly lower from those obtained for all other tissues under analysis (Figure 3.9).



**Figure 3.9.** Effect of IL-1 $\beta$  on COX-2 expression. Quiescent fibroblasts from control-NM (n = 6), control-BM (n = 3), NM-AIA (n = 4), NP-AIA (n = 5), and BM-AIA (n = 3) were incubated with 10ng/mL IL-1 $\beta$  for 24 hours. \*  $p < 0.05$  compared with control-NM fibroblasts.

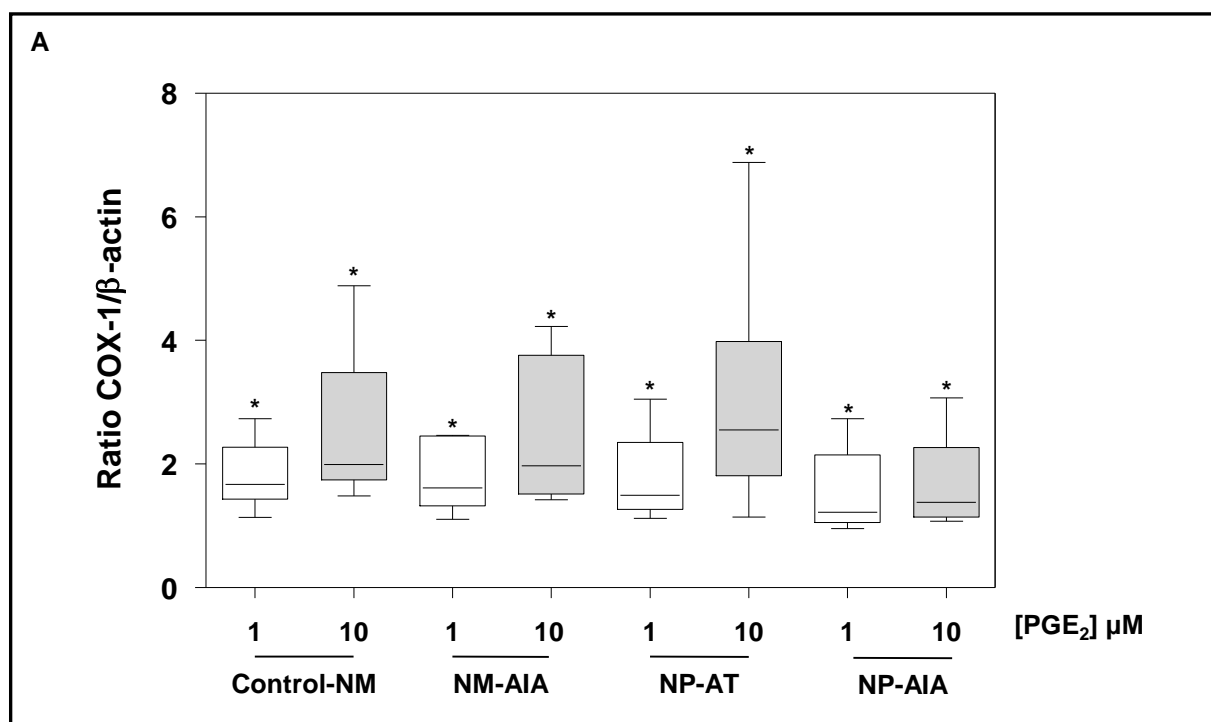
### 3.5.3. Effect of PGE<sub>2</sub> on COX Expression

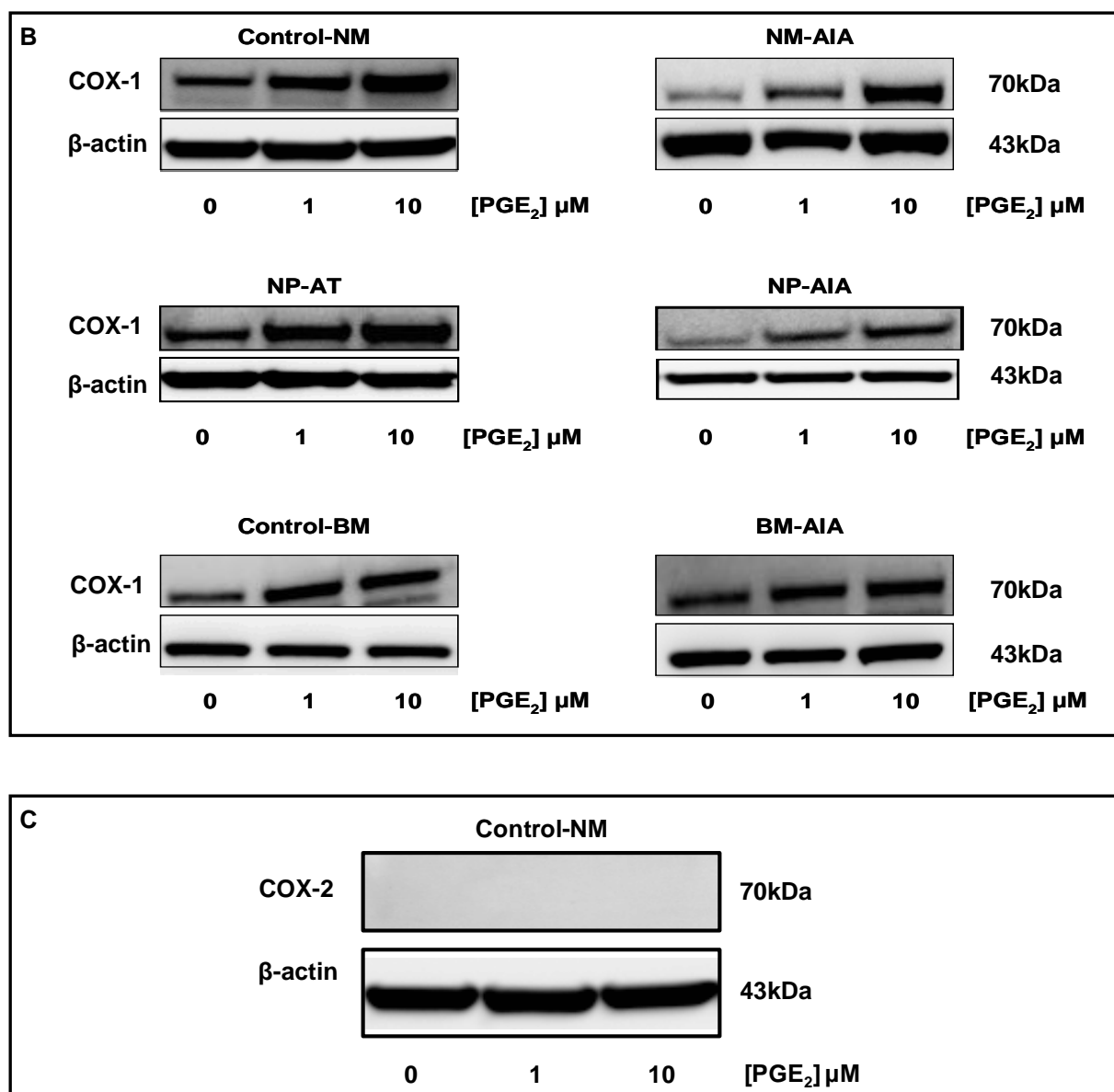
To evaluate the effect of PGE<sub>2</sub> in both COX-1 and COX-2 protein expression, quiescent fibroblasts from control-NM, NP-AT, control-BM, NM-AIA, NP-AIA, and BM-AIA, were incubated with SFM in the absence or presence of 1 or 10 $\mu$ M PGE<sub>2</sub> for 24 hours. Compared with cells incubated with culture media, COX-1 protein expression was significantly increased in fibroblasts from control-NM (1 $\mu$ M = 1.67; 1.44-2.27, 10 $\mu$ M = 1.99; 1.74-3.48), NM-AIA (1 $\mu$ M = 1.61; 1.33-2.45, 10 $\mu$ M = 1.97; 1.52-3.76), NP-AT (1 $\mu$ M = 1.49; 1.28-2.35, 10 $\mu$ M = 2.55; 1.81-3.98), and NP-AIA (1 $\mu$ M = 1.22; 1.06-2.15, 10 $\mu$ M = 1.38; 1.14-2.27) when cells were incubated with 1 or 10 $\mu$ M PGE<sub>2</sub> (Figure 3.10A and B). No statistical differences were found when the different fibroblast cultures were compared.

The effect of PGE<sub>2</sub> on COX-1 protein expression was also evaluated in cultured fibroblasts from both control-BM and BM-AIA. In these samples the stimulation of fibroblasts with 1 or 10 $\mu$ M PGE<sub>2</sub> appears to increase the expression of COX-1, such as occurred in fibroblasts from upper airways samples (Figure 3.10B). However, the slight increase found in the expression of COX-1 in these tissues was

not statistically significant, probably due to the low number of samples ( $n = 3$ ) available to perform the experimental protocols.

Additionally, the effect of  $\text{PGE}_2$  on COX-2 protein expression was analyzed. In contrast to the effect found in COX-1 expression,  $\text{PGE}_2$  did not stimulate the expression of COX-2 in any fibroblast cultures. The Figure 3.10C shows a representative immunoblot performed from the same samples used to analyze COX-1 protein expression. The absence of bands in the immunoblot assay clearly demonstrates the lack of detection of COX-2 protein expression when fibroblasts were stimulated with  $\text{PGE}_2$  at different concentrations.





**Figure 3.10.** Effect of PGE<sub>2</sub> on COX enzymes expression. Quiescent fibroblasts from control-NM (n = 10), NM-AIA (n = 7), NP-AT (n = 6), NP-AIA (n = 9), control-BM (n = 3), and BM-AIA (n = 3) were incubated with or without 1 or 10 μM PGE<sub>2</sub> for 24 hours. **A**, COX-1 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot of control-NM, NM-AIA, NP-AT, NP-AIA, control-BM, and BM-AIA, showing the concentration-dependent increase of COX-1 protein expression. **C**, Representative immunoblot of control-NM (same samples used to evaluate COX-1 protein expression), showing the lack of COX-2 protein expression. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with respective control media (COX-1/β-actin ratio = 1).

### 3.6. EP Receptors Expression

#### 3.6.1. Basal Expression of EP Receptors

To evaluate the basal expression of EP receptor subtypes (EP1 – EP4), quiescent fibroblasts were incubated with SFM for 24 hours and expression of proteins was measured by means of immunoblot analysis. The EP1 receptor has a molecular weight of 41 kDa, approximately. When the results obtained from immunoblot were analyzed, non-specific bands were observed on the membranes. Since the molecular weight of these bands did not correspond to the molecular weight of the EP1 receptor, it was not possible to evaluate the basal expression of EP1 receptor in fibroblasts from control-NM, NM-AIA, NP-AT, NP-AIA, control-BM, and BM-AIA tissues. On the other hand, the baseline expression of EP2, EP3, and EP4 receptors was measured, and the results obtained from statistical analysis are shown in Table 3.1.

EP2 protein expression was found in all fibroblast cultures. At baseline, there were no differences in EP2 protein expression when fibroblasts from control-NM and NM-AIA were compared. However, compared to control-NM, the basal expression of EP2 in fibroblasts from NP-AT was significantly higher. In addition, the expression of EP2 in fibroblasts from NP-AIA was significantly lower compared with both control-NM and NP-AT (Figure 3.11).

On the other hand, there were no differences in EP3 protein expression when comparing fibroblasts from control-NM with fibroblasts from NP-AIA. However, the EP3 expression was significantly lower in fibroblasts from NP-AT and NM-AIA tissues, when comparing with both control-NM and NP-AIA. Additionally, the basal expression of EP3 in fibroblasts from NM-AIA was significantly lower compared with both NP-AT and NP-AIA (Figure 3.12).

At baseline, there were no significant differences in the expression of EP4 in cultured fibroblasts from control-NM, NM-AIA, and NP-AT. Nevertheless, it was observed a higher expression of this receptor among cells from NP-AIA tissue, which was significantly different, when compared with control-NM, NM-AIA, and NP-AT (Figure 3.13).

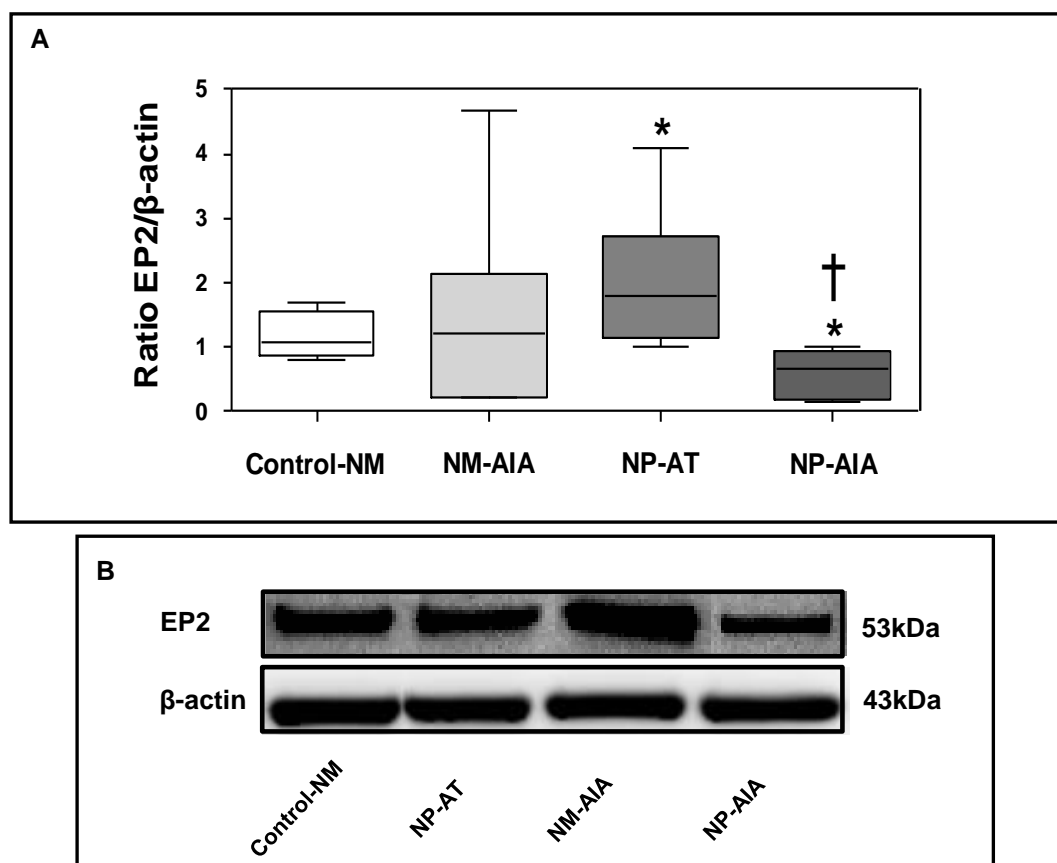
The basal expression of the EP receptors was also measured in cultured fibroblasts from both BM (control and AIA). The results were not represented in Table 3.1., since the number of samples available to perform the protocols was too small. Moreover, the results showed a great variability. However, when the statistical analysis was performed, differences in the expression of EP3 and EP4 receptors were found, but not in EP2 receptor. At baseline, the expression of EP3 seems to be lower in fibroblasts from control-BM when compared with control-NM and both NP-AT and NP-AIA (Figure 3.12B). Considering the baseline expression of EP4 receptor, the results demonstrated that this receptor could be lower in fibroblasts from control-BM, when compared to NP-AIA, as was verified for the other tissues (Figure 3.13B).



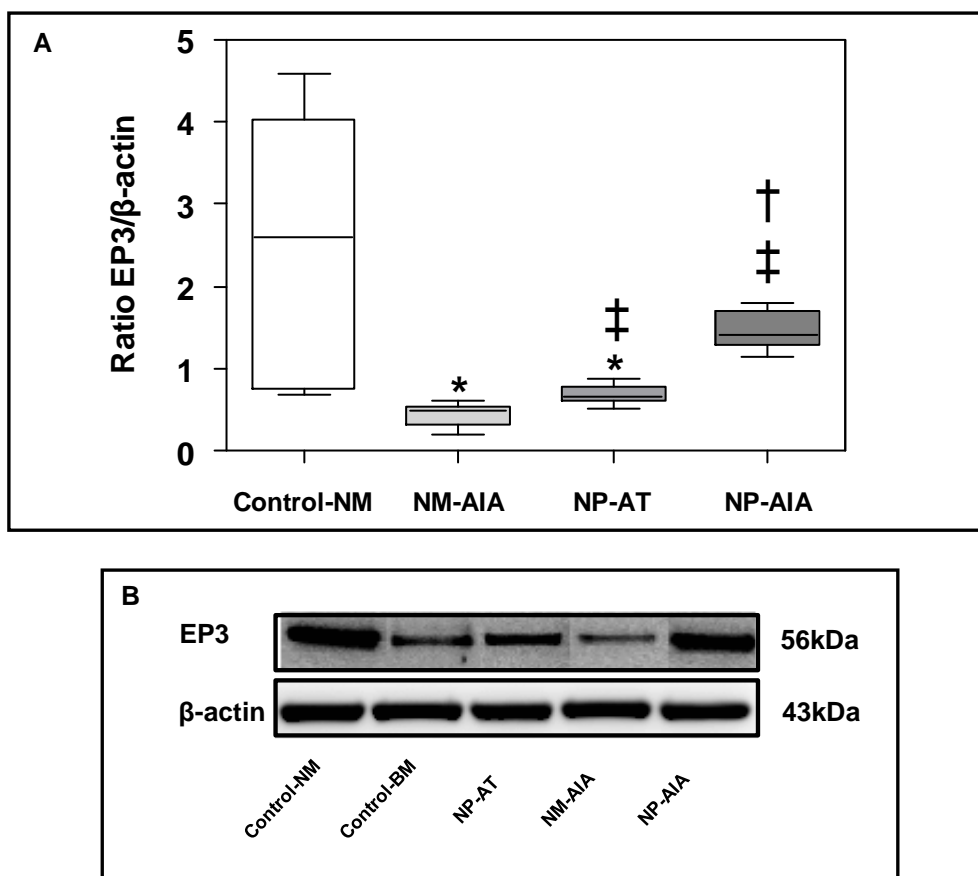
**Table 3.1.** Baseline levels of EP receptors in cultured fibroblasts.

| EP receptor | Control-NM (n=7)    | NM-AIA (n=8)         | NP-AT (n=9)            | NP-AIA (n=6)            |
|-------------|---------------------|----------------------|------------------------|-------------------------|
| EP1         | Not detected        | Not detected         | Not detected           | Not detected            |
| EP2         | 1.07<br>(0.87-1.56) | 1.22<br>(0.21-2.14)  | 1.18*<br>(1.14-2.72)   | 0.67 *†<br>(0.18-0.93)  |
| EP3         | 2.60<br>(0.75-4.03) | 0.49*<br>(0.31-0.55) | 0.65 *‡<br>(0.62-0.77) | 1.41 ‡†<br>(1.30-1.70)  |
| EP4         | 0.34<br>(0.27-0.54) | 0.87<br>(0.25-1.16)  | 0.44<br>(0.34-0.70)    | 1.64 *‡†<br>(1.18-2.21) |

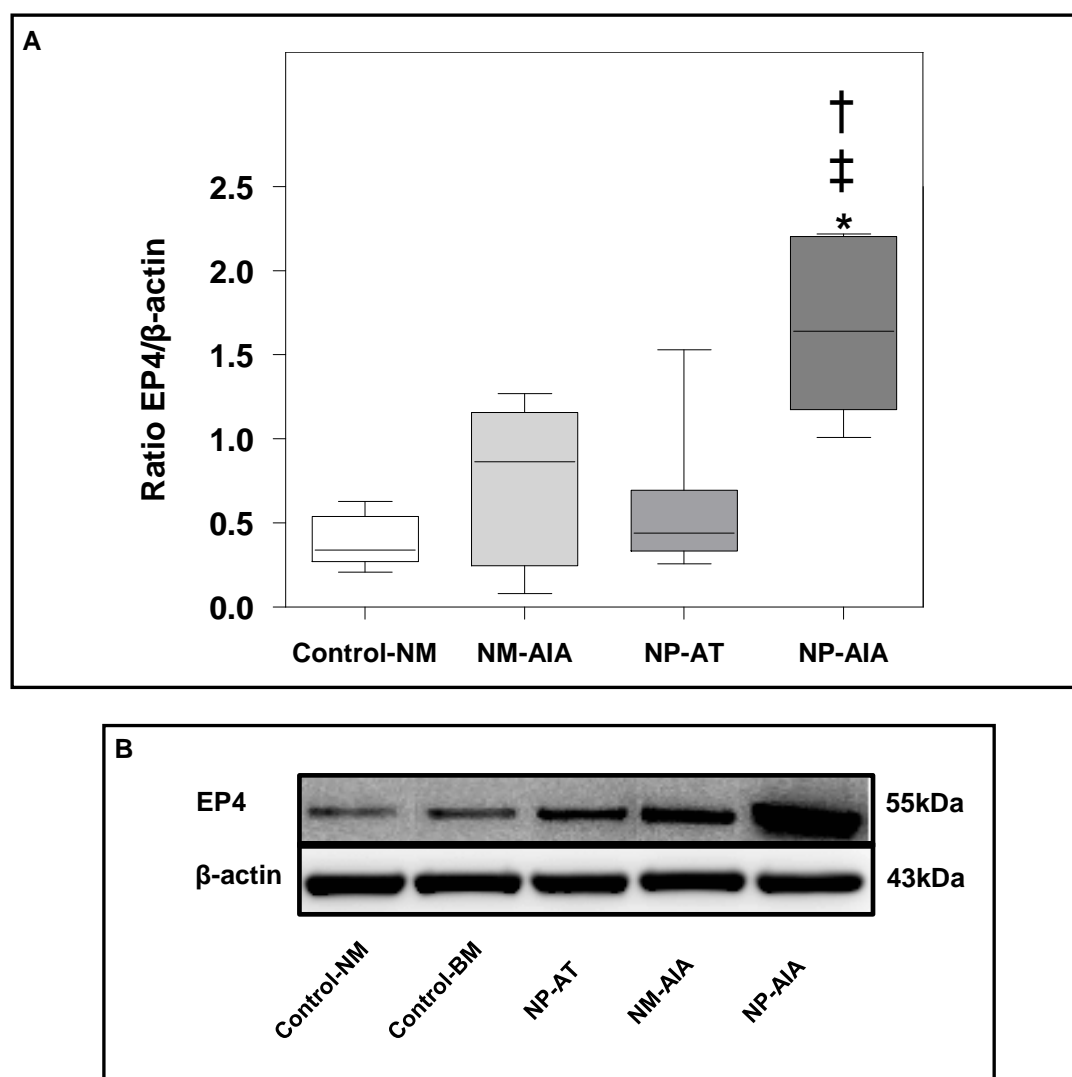
The results are expressed as medians (25<sup>th</sup> to 75<sup>th</sup> interquartiles) of the EP receptor/ $\beta$ -actin ratio. The Mann-Whitney *U* test was used for comparisons between groups. \*  $p < 0.05$  compared with control-NM. ‡  $p < 0.05$  compared with NM-AIA. †  $p < 0.05$  compared with NP-AT.



**Figure 3.11.** Basal expression of EP2 receptor on cultured fibroblasts. Quiescent fibroblasts from control-NM ( $n = 7$ ), NM-AIA ( $n = 8$ ), NP-AT ( $n = 9$ ), NP-AIA ( $n = 6$ ), control-BM ( $n = 4$ ), and BM-AIA ( $n = 4$ ) were incubated for 24 hours with SFM. **A**, EP2 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot of control-NM, NP-AT, NM-AIA, and NP-AIA, showing the differences between tissues. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control-NM. †  $p < 0.05$  compared with NP-AT.



**Figure 3.12.** Basal expression of EP3 receptor on cultured fibroblasts. Quiescent fibroblasts from control-NM ( $n = 7$ ), NM-AIA ( $n = 8$ ), NP-AT ( $n = 9$ ), NP-AIA ( $n = 6$ ), control-BM ( $n = 4$ ), and BM-AIA ( $n = 4$ ) were incubated for 24 hours with SFM. **A**, EP3 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot of control-NM, control-BM, NP-AT, NM-AIA, and NP-AIA, showing the differences between tissues. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control-NM. ‡  $p < 0.05$  compared with NM-AIA. †  $p < 0.05$  compared with NP-AT.



**Figure 3.13.** Basal expression of EP4 receptor on cultured fibroblasts. Quiescent fibroblasts from control-NM ( $n = 7$ ), NM-AIA ( $n = 8$ ), NP-AT ( $n = 9$ ), NP-AIA ( $n = 6$ ), control-BM ( $n = 4$ ), and BM-AIA ( $n = 4$ ) were incubated for 24 hours with SFM. **A**, EP4 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot of control-NM, control-BM, NP-AT, NM-AIA, and NP-AIA, showing the differences between tissues. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control-NM. ‡  $p < 0.05$  compared with NM-AIA. †  $p < 0.05$  compared with NP-AT.

### 3.6.2. Effect of IL-1 $\beta$ on EP Receptors Expression

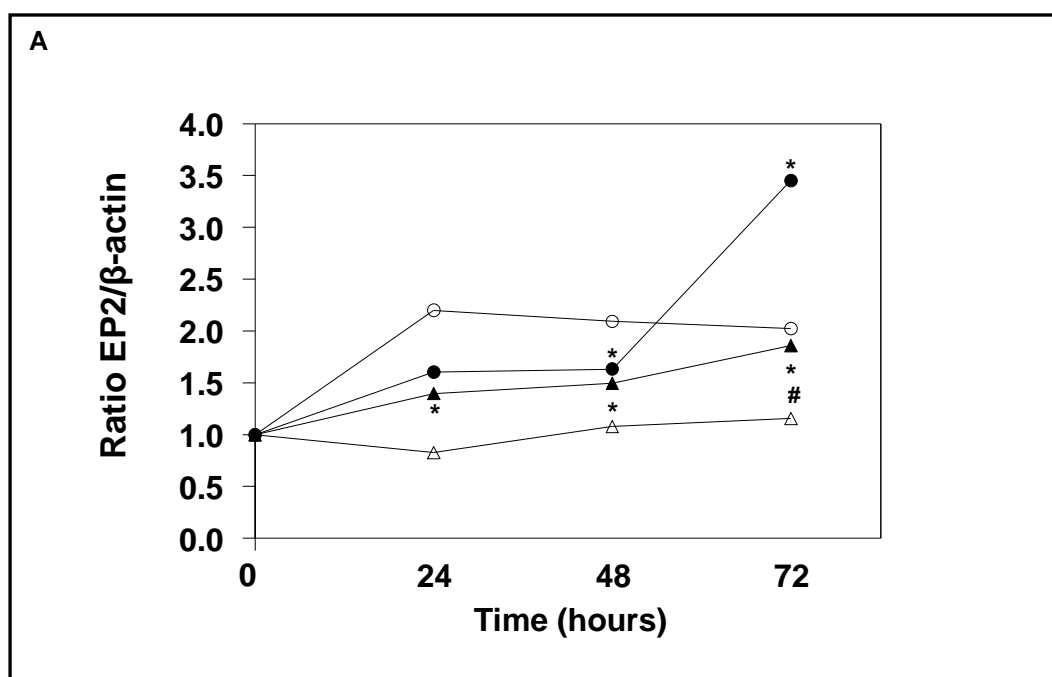
In order to evaluate the effect of IL-1 $\beta$  on EP receptors expression (EP1 – EP4), quiescent fibroblasts were incubated with or without the cytokine and EP protein expression was measured by means of immunoblot at 0, 24, 48, and 72 hours. As seen in basal expression of EP receptors, it was not possible to detect EP1 receptor when cultured fibroblasts were stimulated with 10ng/mL IL-1 $\beta$  for 72 hours.

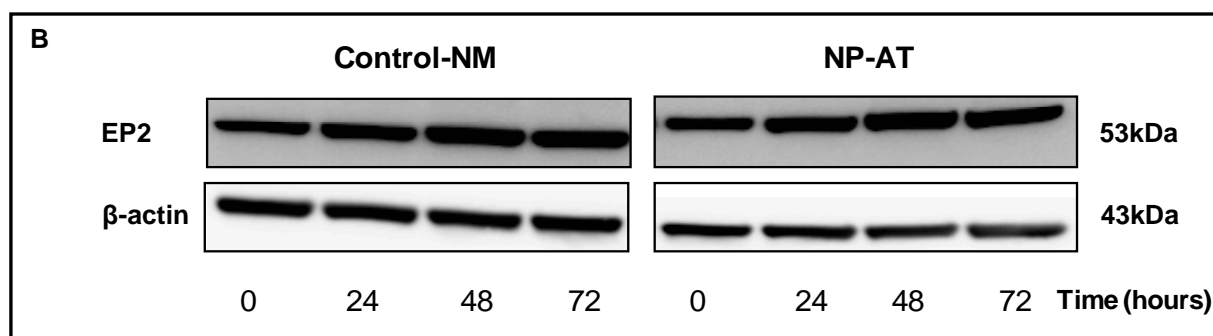
The EP2 protein expression increased significantly in control-NM and NP-AT when fibroblasts were incubated with 10ng/mL IL-1 $\beta$  at 24 (NP-AT = 1.40; 1.30-1.62), 48 (control-NM = 1.87; 1.29-2.67, NP-AT = 1.50; 1.19-2.05), and 72 hours (control-NM = 3.66; 1.61-4.25, NP-AT = 1.86; 1.45-2.26), when compared with the respective control media (Figure 3.14A). The expression of EP2 receptor in fibroblasts from control-NM increased as early as 24 hours (control-NM = 2.02; 1.17-2.63), when cells were stimulated with IL-1 $\beta$ , but this increase was not statistically significant ( $p = 0.06$ ) (Figure 3.14B). The stimulation of fibroblasts with 10ng/mL IL-1 $\beta$  did not increase the expression of EP2 in fibroblasts from NM-AIA and NP-AIA, when comparing with control media. Additionally, at 72 hours, the expression of EP2 receptor was significantly lower in fibroblasts from NP-AIA tissue (NP-AIA = 1.16; 0.78-1.99), when compared with control-NM (Figure 3.14A).

The expression of EP3 was significantly increased in cultured fibroblasts from NP-AIA incubated with 10ng/mL IL-1 $\beta$  at 48 (NP-AIA = 1.38; 1.13-1.60) and 72 (NP-AIA = 1.37; 0.95-1.79) hours, when comparing with the respective control media. Besides, the expression of EP3 receptor was also significantly increased in fibroblasts from NP-AIA tissue at 24 and 48 hours, when compared with control-NM (control-NM at 24 hours = 0.91; 0.77-1.11, control-NM at 48 hours = 0.82; 0.71-1.37). The incubation of fibroblasts from control-NM, NM-AIA, and NP-AT with IL-1 $\beta$  did not alter the expression of EP3 receptor (Figure 3.15A and B).

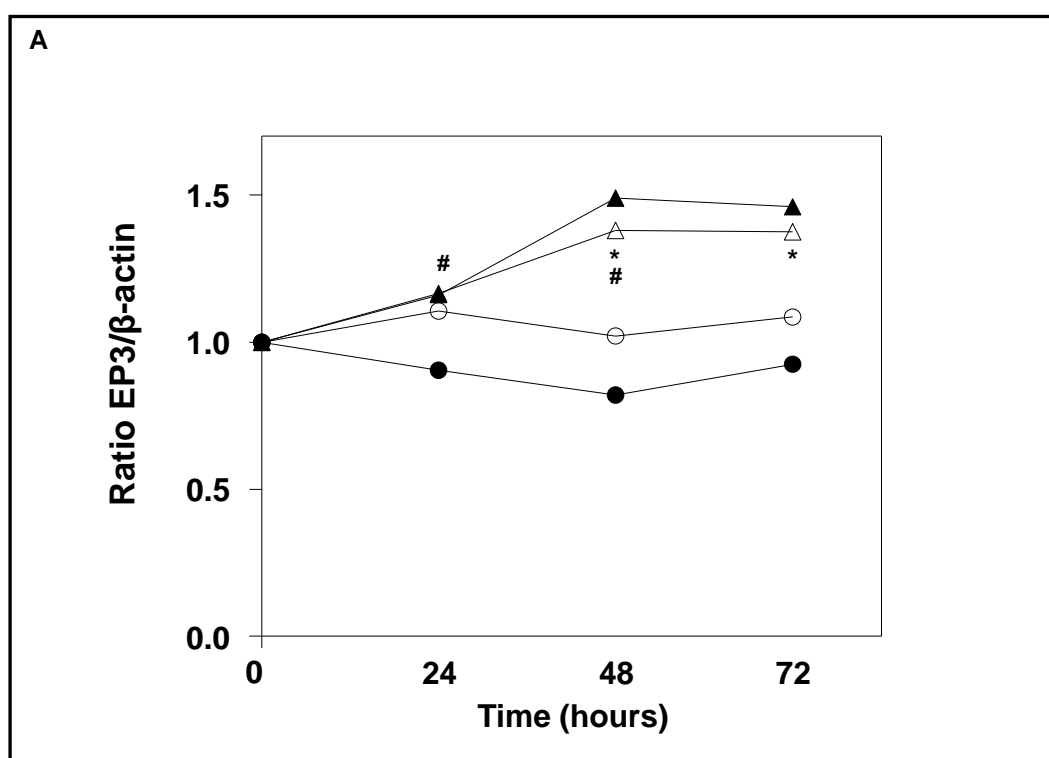
Relatively to the EP4 receptor, the results show that the incubation of fibroblasts with 10ng/mL IL-1 $\beta$  did not change the expression of EP4 in any fibroblast cultures.

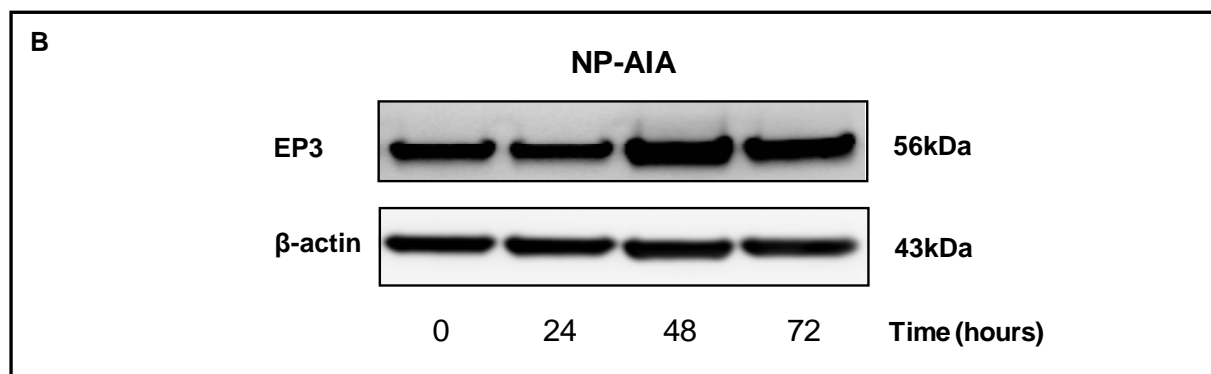
The effect of IL-1 $\beta$  on the expression of EP receptors was also evaluated in fibroblasts from both control-BM and BM-AIA. The results demonstrate that in these cells the stimulation with the pro-inflammatory cytokine doesn't appears to modify the expression of any receptors (EP2, EP3 or EP4).





**Figure 3.14.** Effect of IL-1 $\beta$  on EP2 protein expression. Quiescent fibroblasts from control-NM ( $n = 7$ ), NP-AT ( $n = 9$ ), NM-AIA ( $n = 4$ ), and NP-AIA ( $n = 9$ ) were incubated with 10ng/mL IL-1 $\beta$  for 72 hours. **A**, EP2 expression in fibroblasts from control-NM (black circle), NM-AIA (white circle), NP-AT (black triangles), and NP-AIA (white triangles) was analyzed by means of immunoblotting. **B**, Representative immunoblots of control-NM and NP-AT showing the time-dependent increase in EP2 expression. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control media (EP2/ $\beta$ -actin ratio = 1). #  $p < 0.05$  compared with control-NM fibroblasts incubated with IL-1 $\beta$ .





**Figure 3.15.** Effect of IL-1 $\beta$  on EP3 protein expression. Quiescent fibroblasts from control-NM (n = 8), NP-AT (n = 7), NM-AIA (n = 4), and NP-AIA (n = 10) were incubated with 10ng/mL IL-1 $\beta$  for 72 hours. **A**, EP3 expression in fibroblasts from control-NM (black circle), NM-AIA (white circle), NP-AT (black triangles), and NP-AIA (white triangles) was analyzed by means of immunoblotting. **B**, Representative immunoblot of NP-AIA showing the time-dependent increase in EP3 expression. The molecular weight of each protein is also represented. \*  $p < 0.05$  compared with control media (EP3/ $\beta$ -actin ratio = 1). #  $p < 0.05$  compared with control-NM fibroblasts incubated with IL-1 $\beta$ .





## 4. Discussion

According to the recent concept of “united airways” (also known as “one airway, one disease”), inflammatory processes of the upper and lower airways often coexist and share some mechanisms (Guilemany *et al.*, 2008). Effectively, the histology and physiology of the upper and lower respiratory tracts show many similarities and some differences. In addition, the inflammatory diseases that affect the upper airways (rhinitis and sinusitis) share some common physiopathological mechanisms with those affecting the lower airways (asthma). In this study, it was investigated the different components of the COX pathway, as the secretion of PGE<sub>2</sub> and the expression of both COX enzymes and also the expression of EP1 through EP4 receptors in cultured fibroblasts isolated from NM and NP (upper airways) and BM (lower airways) tissues from patients without asthma who are tolerant to aspirin, patients with asthma who are intolerant to aspirin, and control subjects. The main objective of this investigation was to establish a comparison between the upper and lower airways of these subjects, and to verify if there are differences between them, to contribute for a best understanding of the metabolism of AA through the COX pathway, and the possible abnormalities that are present at this level.

The main findings of this study are: **1)** IL-1 $\beta$  stimulation markedly increased the release of PGE<sub>2</sub> in fibroblasts from control-NM, control-BM, and BM-AIA. Additionally, IL-1 $\beta$  has a much less stimulatory effect in NP-AIA samples and non-significant effect on NM-AIA samples. **2)** Stimulation of fibroblasts with IL-1 $\beta$  increased the expression of COX-2 in both BM samples (control and AIA). However, the expression of COX-2 in stimulated fibroblasts from NP-AIA samples was almost undetectable. **3)** The baseline levels of COX-1 were higher in both BM samples (control and AIA) compared with upper airways. **4)** Stimulation of fibroblasts with IL-1 $\beta$  increased the expression of COX-1 in control-NM, but not in stimulated fibroblasts from NM-AIA, NP-AT and NP-AIA. **5)** Stimulation of fibroblasts with PGE<sub>2</sub> increased the expression of COX-1 in all tissues from upper airways, without significant differences. **6)** The expression level of the EP2 receptor was low in fibroblasts from NP-AIA, while it was high in fibroblasts isolated from NP-AT tissues. **7)** The expression of EP3 was low in fibroblasts from NP-AT and NM-AIA tissues, and high in fibroblasts from NP-AIA, after stimulation with IL-1 $\beta$ . **8)** The expression of EP4 receptor was increased in fibroblasts from NP-AIA tissues.

### 4.1. PGE<sub>2</sub> Secretion

PGE<sub>2</sub> seems to play an important role in inflammatory processes. This prostanoid is commonly considered a potent pro-inflammatory mediator, but in the lungs PGE<sub>2</sub> exerts several anti-inflammatory and anti-fibrotic effects, that include, for instance, attenuation of eosinophil infiltration, reduction of Cys-LTs release, inhibition of fibroblast proliferation, myofibroblast transformation and collagen synthesis (Vancheri *et al.*, 2004). In the present investigation, it was reported a markedly increase in

the secretion of PGE<sub>2</sub> in fibroblasts from control-NM, control-BM, and BM-AIA, after cell incubation with a pro-inflammatory cytokine (IL-1 $\beta$ ), for 24 hours. Moreover, the cytokine had a slight, but significant effect, in the secretion of PGE<sub>2</sub> in fibroblasts from NP-AIA tissues. This last result is in agreement with previous studies that demonstrated decreased production of PGE<sub>2</sub> in NPs, which is more accentuated in NPs of patients who suffer from AIA. Altered secretion of PGE<sub>2</sub> is a characteristic of NPs and AIA and is probably due to an abnormal regulation of the AA pathway in patients with NPs especially among those with AIA (Yoshimura *et al.*, 2008; Pérez-Novo *et al.*, 2005; Pierzchalska *et al.*, 2003). However, it seems to exist some contradictory results when PGE<sub>2</sub> secretion is analyzed: *in vitro* studies have demonstrated reduced secretion of PGE<sub>2</sub> in several cell types (Jedrzejczak-Czechowicz *et al.*, 2008; Pierzchalska *et al.*, 2003; Kowalski *et al.*, 2000), but *in vivo* studies reported discrepant results, with high, similar or lower levels of PGE<sub>2</sub> in nasal or bronchoalveolar fluids from patients who suffer from AIA, compared with AT asthmatic patients or control subjects (Gyllfors *et al.*, 2003; Langmack and Wenzel, 1998; Szczeklik *et al.*, 1996; Picado *et al.*, 1992). In BM of patients who suffer from AIA, the production of PGE<sub>2</sub> is not altered, and the values observed at this level are similar to those found in NM or BM of control subjects, after stimulation with the pro-inflammatory cytokine. Effectively there is an altered production of PGE<sub>2</sub> in upper airways, but this alteration was not verified in lower airways. As previously described, polyps are inflammatory structures that are never seen in the lower airways. On the basis of these observations, the differential secretion of PGE<sub>2</sub> in upper and lower airways can be related with the presence or absence of polyps, i.e., can be involved in the pathogenesis of nasal polyposis.

## 4.2. COX Enzymes Expression

The induction of COX-2 under inflammatory conditions leads to the synthesis of several PGs, and one of them is the PGE<sub>2</sub> (Baigent and Patrono, 2003). Studies that investigate the expression of COX-2 show some discrepancies and Pujols *et al.* reported that the differences between publications may be due to the study methods (methodology used to perform the experiments and technical limitations of some experimental protocols) (Pujols *et al.*, 2004). In this study, it was reported that the stimulation of fibroblasts with a pro-inflammatory cytokine, for 24 hours, increased the expression of COX-2 in both BM (control and AIA) without differences, when comparing with control-NM. Moreover, in the same conditions, the expression of COX-2 in fibroblasts from NP-AIA is almost undetectable. This last result is in agreement with previous studies that observed either reduced levels of COX-2 mRNA in NPs, especially in patients who suffer from AIA (Pujols *et al.*, 2004) and reduced levels of COX-2 protein in fibroblasts from NP-AIA tissues (Roca-Ferrer *et al.*, 2011). The increased expression of COX-2 verified in lower airways of control subjects and patients who suffer from AIA might contribute to the high secretion of PGE<sub>2</sub> observed in the lungs, since COX-2 is the major source to the production of PGE<sub>2</sub>. Considering that, and the results reported for PGE<sub>2</sub> in this investigation, the COX pathway seems to be differentially regulated when upper and lower airways are compared, and in lower airways the presence of aspirin intolerance does not modify the expression of COX-2 or the secretion

of PGE<sub>2</sub>, since the levels in both control individuals and patients who suffer from AIA were not different.

COX-1 is considered an enzyme with a constitutive expression in a broad range of cells and tissues, where it mediates the synthesis of PGs required for physiological functions. Although COX-1 responds to the physiological needs of cells, this enzyme is not considered to be involved in inflammatory responses (Simmons *et al.*, 2004). Some studies found an altered expression of this enzyme in airways of patients with aspirin sensitivity (Pierzchalska *et al.*, 2007; Pujols *et al.*, 2004; Pierzchalska *et al.*, 2003), and other did not report any differences (Picado *et al.*, 1999). Since the knowledge about the expression of COX-1 enzyme is very limited and little is known about its regulation, the basal expression of COX-1 was analyzed in this study. At baseline, there were no differences between patients with or without aspirin sensitivity and control subjects, but it was reported higher levels of COX-1 in both BM (control and AIA) without differences, when compared with upper airways. Considering these results, it was demonstrated that COX-1 has a differential regulation in upper and lower airways and the aspirin sensitivity does not appears to be a factor that alters the regulation of the COX-1 enzyme in lower airways, since the expression of COX-1 was not different between control subjects and patients who suffer from AIA, in fibroblasts from both BM.

Some studies reported small increases in the expression of COX-1 in inflamed tissues (McAdam *et al.*, 2000). For instance, in NPs from patients with CRS associated with cystic fibrosis COX-1 was reported to be up-regulated, indicating that airway COX-1 is sensitive to inflammatory stimuli (Roca-Ferrer *et al.*, 2006). Unfortunately, studies that measured expression of COX-1 in both control-NM and NPs of patients with or without aspirin intolerance showed contradictory results: some did not found any differences in COX-1 expression between NM and NPs (Adamjee *et al.*, 2006; Picado *et al.*, 1999), but others found higher levels of this enzyme in NPs from patients with AIA compared with control subjects (Pujols *et al.*, 2004). In bronchial epithelial cells from asthmatic patients and control subjects it was reported lower levels of COX-1 mRNA in asthmatic patients, and this abnormal event is even more accentuated in patients with AIA (Pierzchalska *et al.*, 2007). Previous studies found a delay between transcription of mRNA and increased levels of COX-1 protein (Bunimov *et al.*, 2007; Kang *et al.*, 2007). Additionally, COX-1 protein has a very long half-life (Bunimov *et al.*, 2007; Kang *et al.*, 2007). According with these observations, the expression of COX-1 protein was measured up to 72 hours after fibroblast stimulation with a pro-inflammatory cytokine. The results show increased levels of COX-1 in control-NM tissue, but not in NM-AIA or NPs with or without aspirin intolerance. Altered expression of COX-1 in patients who suffer from NP combined with AIA might contribute to the low levels of PGE<sub>2</sub> detected in patients with NP-AIA. In fibroblasts from BM tissues, the stimulation with the pro-inflammatory cytokine appears to increase the expression of COX-1 in both control subjects and AIA patients, without differences, as previously reported at baseline. Since previous studies observed alterations in the regulation of COX-1 in fibroblasts from patients with AIA, similar results were expected in BM-AIA. However this was not verified. Despite, the number of samples to evaluate the expression of COX-1 in fibroblasts from BM was limited, there was a clearly increase in its

expression after stimulation with the pro-inflammatory cytokine. To confirm the results it would be necessary to increase the number of samples, and probably performed different techniques.

There has been accumulating evidence demonstrating the regulatory role of PGs in inflammation. In particular, a number of studies focused on the feedback control of COX-2 (Vichai *et al.*, 2005). However, the results presented in these investigations have been varied and largely dependent of the cell type. Studies using human synovial fibroblasts, monocytes and the prostatic carcinoma cell line (PC-3) showed positive effects of PGE<sub>2</sub> on COX-2 (Faour *et al.*, 2001; Hinz *et al.*, 2000; Tjandrawinata and Hughes-Fulford, 1997), whereas a study using the human umbilical vein endothelial cell line showed that PG downregulated COX-2 protein synthesis (Akarasereenont *et al.*, 1999). A previous study performed in mouse lung fibroblasts reported a positive feedback on the regulation of COX-2 expression by PG metabolites, as PGE<sub>2</sub>, in the presence of an inflammatory stimulus, but not on the regulation of COX-1 expression (Vichai *et al.*, 2005). The same authors hypothesized that the induction of COX-2 protein expression by both PGE<sub>2</sub> and IL-1 $\beta$  might result in the activation of different pathways and that a product of IL-1 $\beta$ -responsive gene might enhance COX-2 inducing PGE<sub>2</sub> activity. On the basis of these observations and in order to analyze if PGE<sub>2</sub> alone, i.e., in the absence of a pro-inflammatory stimulus, induce the expression of COX-1 and COX-2 enzymes, fibroblasts were incubated with PGE<sub>2</sub>, for 24 hours, and the expression of both enzymes was measured. The results showed that PGE<sub>2</sub> is able to increase alone the expression of COX-1 enzyme, without differences between the tissues under analysis, i.e., there are no differences between control subjects, AT and AIA patients. These findings suggest the presence of a mechanism by which PGE<sub>2</sub> regulates the expression of COX-1, without the presence of an inflammatory stimulus. On the other hand, when COX-2 was analyzed, it was not detected any expression of the enzyme. Considering these results, it is possible that COX-2 protein is only expressed under inflammatory conditions, and the PGE<sub>2</sub> alone is not able to induce the expression of this enzyme. Moreover, it is possible that the concentrations of PGE<sub>2</sub> used in this study regulate negatively the expression of COX-2. However, the multiplicity of components involved in this signalling pathway probably complicates the process of regulation, and more studies are necessary to confirm these findings.

### 4.3. EP Receptors Expression

PGE<sub>2</sub> is normally considered a potent pro-inflammatory mediator, actively involved in the pathogenesis of several diseases. However, in the lungs, PGE<sub>2</sub> has functions that reduce airway hyper-responsiveness and inflammation, as well as that control tissue repair (Vancheri *et al.*, 2004). The ability of PGE<sub>2</sub> to induce or suppress several mechanisms involved in inflammation indicates the complex activities of its receptors (Kang *et al.*, 2007). The EP receptors (EP1-EP4) signal through distinct pathways and the expression of their genes is regulated by various physiological and pathophysiological stimuli (Sugimoto and Narumiya, 2007). Considering these data, it was

investigated the levels of each EP receptor in fibroblasts, at baseline and after cell stimulation by the action of a pro-inflammatory stimulus, since some receptors required cell activation.

At baseline and after cell stimulation with a pro-inflammatory cytokine, the expression of EP1 receptor was not detected. The lack of detection can be due to the low expression of this receptor in the cells used to perform the experiment or to the methodology used, which might present some technical limitations. Burgess and collaborators, in a previous study to assess the presence of EP receptors on airway smooth muscle cells isolated from asthmatic patients and non asthmatic individuals, also failed to detect the presence of EP1 receptor by means of ELISA (Burgess *et al.*, 2004). However, there are several studies that reported the presence of this receptor in different cell types, and different methodologies are used to perform the analysis (Ying *et al.*, 2006; Pérez-Novo *et al.* 2006; Roca-Ferrer *et al.*, 2011).

In basal conditions, a previous study (Roca-Ferrer *et al.*, 2011) did not find any differences in the expression of EP1 through EP4 receptors among fibroblasts from control-NM, NP-AT, or NP-AIA tissues. However, Ying and collaborators in a study performed to compare EP receptor expression in aspirin intolerant and tolerant patients showed a reduced number of eosinophils, neutrophils, mast cells and T cells that express EP2 in NP-AIA samples (Ying *et al.*, 2006). The results presented in this study are in agreement with these last findings, since it was reported low basal expression of EP2 in fibroblasts from NP-AIA tissue. Additionally, it was also reported an increase in the expression of EP2 receptor in fibroblasts from NP-AT tissues. Effectively, in a previous study it was demonstrated that EP2 receptor is up-regulated in patients who suffer from CRS with NP compared with control subjects (Pérez-Novo *et al.*, 2006). According with these findings, when fibroblasts are stimulated with a pro-inflammatory cytokine, the expression of EP2 is increased in fibroblasts from control-NM and NP-AT, but not in fibroblasts from NP-AIA. Roca-Ferrer *et al.* in a previous study, reported an increase in EP2 expression in cells from control-NM after stimulation with IL-1 $\beta$ , but not in NP-AT tissues. However, it appears to be increased, although without statistical differences (Roca-Ferrer *et al.*, 2011). Most of the anti-inflammatory effects of PGE<sub>2</sub> are mediated through the EP2 receptor. Considering that, the expression of EP2 in NP-AT samples could be increased as a compensatory mechanism. Additionally, this compensatory mechanism could be not present in NP-AIA samples, since the expression of EP2 receptor in these samples is lower when comparing with control-NM and NP-AT samples.

Signalling through EP3 is known to be both pro-inflammatory and anti-inflammatory. Coupling of PGE<sub>2</sub> to EP3 potentiates mast cell degranulation, which enhances inflammatory processes. Contrary, it has been reported recently that EP3 is involved in the suppression of allergic inflammation in an animal model of asthma (Kim *et al.*, 2007). A previous study showed that EP3 transcripts were significantly decreased in patients with CRS and NP (Pérez-Novo *et al.*, 2006). These findings are in agreement with the results obtained in this study, since at baseline low expression of EP3 was observed. In fibroblasts from NP samples the expression of EP3 receptor was increased after stimulation with the pro-inflammatory stimulus. However, this increase was only significant in fibroblasts from NP-AIA tissues. EP3 receptor is known to have multiple splice variants, which complicate the interpretation of

the different results obtained. However, and considering the high expression of EP3 under inflammatory conditions, this receptor could play a role in upper airway inflammation.

The EP4 receptor appears, in some studies, to play a pro-inflammatory role in the pathogenesis of some diseases, as rheumatoid arthritis. Additionally, mice deficient in the EP4 receptor exhibits an attenuated inflammatory response, since the levels of pro-inflammatory cytokines are significantly reduced, as well as the clinical signs of the disease (Hata and Breyer, 2004). In contrast, a recent study demonstrated that the EP4 receptor is responsible for PGE<sub>2</sub>-induced relaxation of human airway smooth muscle (Buckley *et al.*, 2011), which indicates that this receptor also possesses anti-inflammatory proprieties. Pérez-Novo and collaborators reported that EP4 receptor is up-regulated in subjects who suffer from CRS with NPs compared to control individuals (Pérez-Novo *et al.*, 2006). In this study, it is reported an increased expression of EP4 receptor in fibroblasts from NP-AIA tissues. On the basis of the observations previously presented, the high expression of EP4 in fibroblasts from NP-AIA could represent a compensatory mechanism.

#### **4.4. Limitations of the Study**

This study presents some limitations. Since our experimental model is based on primary cell cultures i.e., explants taken directly from the living organism in order to preserve, as well as possible, the characteristics of each patient, and considering that each cell line is respective to a unique individual, the current investigation was limited, mostly, by the lower number of BM samples available to perform the study, as well as by the high inter-sample variability verified in some results. For these reasons the comparison between upper and lower airways was not always clear. Considering that it would be important to increase the number of samples of both BM tissues (control and AIA), and to perform different techniques in order to minimize the variability and to obtain results more robust about the regulation of the different component of the COX pathway.

## 5. Conclusions and Future Perspectives

New insights concerning inflammatory airway diseases, as asthma, nasal polyposis, CRS, and aspirin intolerance, and their respective mechanisms are important, since these syndromes have a high prevalence, and affect the quality of life of several individuals. This investigation was important since it allowed draw some conclusions about the metabolism of the AA pathway, more specifically, about the components of the COX pathway, i.e. COX enzymes, PGE<sub>2</sub> and its respective receptors. Moreover, for the first time, it was established a comparison between the AA metabolism regulation in the upper and lower respiratory tracts of patients without asthma who are tolerant to aspirin, patients with asthma who are intolerant to aspirin, and control subjects. Some studies reported alterations in COX pathway in upper and lower airways, but others not found any differences. Different methodologies and samples used in each investigation are the most probable cause for these contradictory results. For these reasons, and due to these limitations, the role of COX pathway in the pathogenesis of aspirin intolerance was not completely elucidated, and additional studies are necessary to clarify the mechanisms responsible for the syndrome. It is clear that there are differences between upper and lower airways, and, it is interesting that in lower airway the presence of aspirin intolerance does not seems to alter the regulation of the expression of COX enzymes or the secretion of PGE<sub>2</sub>, as previous studies have shown. Effectively, patients with AIA often suffer from nasal polyposis. However, polyps are never seen in the lower airways of patients with bronchial asthma. In this case, the differential regulation of COX enzymes and PGE<sub>2</sub> may play a role at this level. Additionally, it has been demonstrated that PGE<sub>2</sub> is able to induce the expression of COX-1. So, future studies would be necessary to investigate the signaling pathway by which PGE<sub>2</sub> regulates COX-1. Moreover, and considering that PGE<sub>2</sub> is able to induce COX-1, which EP receptor is involved in this stimulation? Further studies using selective agonists and antagonists of EP receptors would be useful to investigate the EP receptor subtype mediating the PGE<sub>2</sub>-stimulated COX-1.

The effect of eicosanoids in the tissue is greatly dependent of the differential expression of the distinct subtypes of their receptors (Pérez-Novo *et al.*, 2006). Considering this observation it is important to understand the pattern of expression of the EP receptors (EP1 through EP4) in the inflammatory airway diseases, to increase the knowledge about the different actions of PGE<sub>2</sub>. Unfortunately, in this investigation the comparison upper *versus* lower airways was less clear with respect to EP receptors expression, since it was found a great variability in the expression of the EP receptors through the lower airways of patients with the same characteristics (control or AIA). However, in this investigation differences were found in nasal tissues of patients who suffer from aspirin intolerance, compared with AT patients and control subjects. Since the methodology used for the analysis of protein expression failed to establish the comparison between upper and lower respiratory tract it would be interesting to use other techniques to evaluate the pattern of expression of these receptors. The real time PCR technique, for instance, can be used to evaluate and measure the mean levels of EP mRNA expression in the same samples analyzed in this study.

These findings together with further studies on COX enzymes, PGE<sub>2</sub> and EP receptors regulation in control subjects and patients with inflammatory airway diseases may help to establish new diagnostics, and to improve current and explore further therapies for the attenuation of the symptoms or for the treatment of the inflammatory diseases discussed in this investigation.

In summary, this study describes the regulation of the different components of the COX pathway in upper and lower airways of patients without asthma who are tolerant to aspirin, patients with asthma who are intolerant to aspirin, and control subjects. Significant differences in the expression of COX-1 and COX-2 enzymes, as well as in the secretion of PGE<sub>2</sub> were reported when upper and lower airways were compared. The data presented in this investigation suggest that in lower airways, the presence of aspirin intolerance does not seem to alter the expression of COX-1 and COX-2 enzymes or the production of PGE<sub>2</sub>, as verified in upper airways of patients with AIA. These results suggest a differential regulation between upper and lower airways, which can be involved in the pathogenesis of nasal polyposis. Considering the expression of the EP receptors, the data suggest differences through fibroblasts from upper airways tissues. However the significance of the differences in EP expression reported in this study must be confirmed by functional studies using selective agonists or antagonist of these receptors. The differences between upper and lower airways described, combined with others observed in previous studies might contribute to the pathogenesis of nasal polyposis.



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